REMARKS

Claims 1-17, 23-25, 27-47, 49-53, 61-73, 77-86, 103, 105-111, 127, 129-132, 134-136, 138-144, 146-157, and 159-164, have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite due to the recitation of the term "prodrug" in claims 1, 43, 103, 127, 132, 136, and 144. Claims 1-17, 23-25, 27-43, 45-47, 49-54, 61-73, 77-86, 103, 105-111, 127, 129-132, 134-136, 138-144, 146-155, 157, and 159-164 are believed to be pending. Claims 156-162 and 164 also have been rejected under 35 U.S.C. §112, first paragraph.

Applicants respectfully request that the Examiner confirm Claims 44 and 133 were cancelled in the Response filed on April 14, 2004. Applicants believe Claim 54 currently is pending, but was inadvertently deemed cancelled in the Office Action dated July 9, 2004.

Claims 1, 43, 103, 127, 132, 136, and 144 have been amended to delete the term "prodrug". Applicants respectfully reserve the right to file a later application on any subject matter presently unclaimed in the application; however, language directed to "prodrug" is deleted to advance the prosecution of the present application.

The Examiner cites rejection of Claims 156-162 and 164. Claims 156 and 158 are presently cancelled. Pending claims 157, 159-162, and 164, as amended, are directed to various methods for treating disorders by administering an effective amount of a compound in Claim 1. The Examiner finds that Applicants' traversal in the Response filed April 14, 2004 is persuasive, but requires further information to assess the state of the art at the time the invention was filed.

Claim 157 has been amended to recite a method of treating a disorder selected from the group consisting of acute myocardial infarction, asthma, bipolar disorder, cognitive enhancement, cognitive deficits in psychiatric disorders, cutaneous carcinoma, drug abuse, depression, gastrointestinal disorders, inflammation, jet lag, medullary thyroid carcinoma, melanoma, allergic rhinitis, Meniere's disease, migraine, mood and attention alteration, motion sickness, neurogenic inflammation, obsessive compulsive disorder, pain, Parkinson's disease, schizophrenia, seizures, septic shock, Tourette's syndrome, vertigo, and wakefulness, comprising administering an effective amount of a compound of Claim 1. Claims 159-162 and 164 are directed to more specific method claims encompassed within the scope of Claim 157.

Applicants respectfully submit that one versed in the pharmaceutical arts would be able to use the invention in a manner commensurate with the scope of the amended claims. Various

publicly-available articles discuss compounds demonstrating known interaction with histamine-3 (H_3) receptors and their uses support Applicants' assertion. Applicants provide copies of some such references for the convenience of the Examiner and accompanied below by a brief description of each article submitted. Applicants welcome the Examiner's independent review of the literature in view of the claims, as amended, and request that the Examiner note all references that are made of record.

Clapham, in the *European Journal of Pharmacology* 259 (1994) 107-114, describes use of a H_3 receptor antagonist for drug-induced hyperactivity.

Lamberti, et al., in the *British Journal of Pharmacology* (1998) 123, 1331-1336, investigate use of H_3 receptor for agonists for treatment of depression.

Pan, et al., in *Meth. Find. Exp. Clin. Pharmacol.* 1998, 20(9): 771-777, describe use of H_3 receptor antagonists for vertigo.

O'Neill, et al., in *Methods Find. Exp. Clin. Pharmacol.* 1999, 21(4): 285-289, describe use of GT-2016, a H_3 receptor antagonist, for vertigo.

Malmberg-Aiello, et al., in the *Br. J. Pharmacology* (1994), 111, 1269-1279, describe the use of histamine-3 receptor ligands for pain.

Prast, et al., in *Brain Research* 734 (1996) 316-318, investigate use of histamine-3 ligands to facilitate social memory and memory deficit.

Chen, et al., in *Brain Research* 839 (1999) 186-189, investigate use of a H_3 receptor antagonist to improve social memory.

Rodrigues, et al., in *Journal of Pharmacology*, (1995) 114, 1523-1524, identify that effective treatment-resistant schizophrenia therapy, particularly clozapine, demonstrates high affinity for histamine-3 receptor binding. Browman, et al., in *Behavioural Brain Research*, 153 (2004) 69-76, further demonstrate the anti-psychotic potential of H_3 receptor ligands for disorders such as schizophrenia.

Panula, et al., in *Neuroscience*, Vol. 82, No. 4, pp. 993-997, associate neuronal histamine deficit in Alzheimer's patients to cognitive decline. Meguro, et al., in *Pharmacology Biochemistry and Behavior*, Vol. 50, No. 3, pp. 321-325 (1995), describe use of histamine-3 antagonists to modulate learning and memory impairment associated with Alzheimer's disease.

Yates, et al., in *The Journal of Pharmacology and Experimental Therapeutics*, Vol. 289, No. 2, describe using H_3 receptor ligands for cognitive disorders associated with attention-deficit disorder.

Ligneau, et al., in *The Journal of Pharmacology and Experimental Therapeutics*, Vol. 287, No. 2, describe using H_3 receptor ligands to modulate sleep disorders and wakefulness.

Yokoyama, et al., in *European Journal of Pharmacology*, 234 (1993) 129-133, investigate H_3 receptor ligands use for inhibiting seizures and convulsions, for examples seizures and convulsions associated with epilepsy.

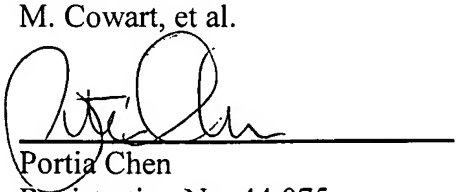
Szelag, in *Medical Science Monitor*, 1998; 4(5): 747-755, describes an inhibitory effect of antagonists of H_3 receptor against proliferation of neosplatic cells of human melanoma, cutaneous carcinoma, and medullary thyroid carcinoma, among others.

McLeod, et al., in *Progress in Respiratory Research*, Basel, Karger, 2001, vol 31, pp. 131-136 and *American Journal of Rhinology*, September-October 1999, Vol. 13, No. 5, describe combination histamine H_1 and H_3 receptor treatment for allergic rhinitis and decongestion.

Applicants respectfully submit that the references, both individually and taken as a whole in context with each other, further clarify and support that the methods described in the application and claimed in Claim 157 are well within the purview of one with skill in the art. As such, it is clear that one with skill in the art would have been able to use the claimed compounds in the claimed methods, particularly in view of demonstrated H_3 receptor binding. Accordingly, Applicants respectfully request that the Examiner withdraw the rejection.

It is respectfully submitted that the present application is in a condition for allowance and notification of the same is respectfully requested.

Respectfully submitted,
M. Cowart, et al.



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APPENDIX

SEVENTEEN (17) REFERENCES

as cited in the

Amendment & Response after Final Rejection

mailed January 8, 2005

ELSEVIER

European Journal of Pharmacology 259 (1994) 107–114

ejp

Thioperamide, the selective histamine H_3 receptor antagonist, attenuates stimulant-induced locomotor activity in the mouse

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Abstract

The effects of the selective histamine H_3 receptor agonist (*R*)-α-methylhistamine and antagonist thioperamide on stimulant-induced locomotor activity in the mouse were examined. Amphetamine (1 mg · kg^{-1} s.c.), apomorphine (2 mg · kg^{-1} s.c.) or cocaine (5 mg · kg^{-1} s.c.) increased locomotor activity. Neither thioperamide (10 mg · kg^{-1} i.p.) nor (*R*)-α-methylhistamine (20 mg · kg^{-1} i.p.) affected spontaneous locomotor activity in their own right. (*R*)-α-Methylhistamine (0.3, 3 or 20 mg · kg^{-1} i.p.) also had no effect on amphetamine (1 mg · kg^{-1} s.c.)-induced locomotor activity. In contrast, thioperamide (0.2–10 mg · kg^{-1} i.p. or 0.3–20 μg i.c.v.) inhibited, in a dose-dependent manner, the hyperactivity response induced by amphetamine (1 mg · kg^{-1} s.c.). (*R*)-α-Methylhistamine (20 mg · kg^{-1} i.p.) completely reversed the inhibitory response to thioperamide (2 mg · kg^{-1} i.p.). Thioperamide (2 or 10 mg · kg^{-1} i.p.) also inhibited apomorphine (2 mg · kg^{-1} s.c.)- and, to a lesser extent, cocaine (5 mg · kg^{-1} s.c.)-induced hyperactivity. We therefore conclude that antagonism of the central histamine H_3 receptor inhibits, to a varying degree, the effects of locomotor stimulants.

Key words: Histamine H_3 receptor; Locomotor activity; (R)-α-Methylhistamine; Thioperamide; (Mouse); (Stimulant)

1. Introduction

There are now many experimental data implicating a role for histamine in arousal mechanisms (see Schwartz et al., 1991). When administered centrally to rodents, histamine is reported to induce biphasic changes in spontaneous locomotor activity. Initially a transitory hypoactivity was observed, followed by a histamine H_1 receptor-mediated hyperactivity (Bristow and Bennett, 1988; Kalivas, 1982). In other studies, however, a hypoactivity response only was obtained, with histamine reducing spontaneous locomotor activity (Alvarez and Guerra, 1982) and inhibiting locomotion induced by methamphetamine (Onodera and Ogura, 1981). In addition, systemic administration of L-histidine, at doses which elevated brain histamine levels (Itoh et al., 1984), also reduced amphetamine- or methamphetamine-induced hyperactivity (Constentin et al., 1974; Itoh et al., 1984). The probable involvement of endogenous histamine is supported by the observation that the histidine decarboxylase inhibitor, α-fluoromethylhistidine, reduced the inhibitory effect of L-histidine (Itoh et al., 1984). Furthermore, metoprine, a histamine-*N*-methyltransferase inhibitor, which increases brain histamine levels, also inhibited methamphetamine-induced hyperactivity (Itoh et al., 1984).

The histamine H_3 receptor is an inhibitory presynaptic heteroreceptor which modulates the release of histamine in the central nervous system (Arrang et al., 1983), and several other neurotransmitters in the central nervous system and periphery (see Timmerman, 1990). Histamine levels in the central nervous system are increased by peripheral administration of the selective histamine H_3 receptor antagonist, thioperamide, but reduced by the selective histamine H_3 receptor agonist, (*R*)-alpha-methylhistamine ((*R*)-α-methylhistamine), also given peripherally (Arrang et al., 1987). Locomotor activity studies with the histamine H_3 receptor agonist and antagonist reveal that the transitory

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hypoactivity induced by central histamine in the rat may be a result of an initial activation of the histamine H_3 receptor (Bristow and Bennett, 1988). In addition, high doses of thioperamide (12.5 and 25 mg · kg^{-1} i.p.) are reported to significantly increase locomotor activity in mast cell-deficient W/W^v mice, an effect that was blocked by pretreatment with (*R*)-α-methylhistamine or mepyramine, a histamine H_1 receptor antagonist, or zolantidine, a histamine H_2 receptor antagonist (Sakai et al., 1991). It was therefore concluded that antagonism of the histamine H_3 receptor increased locomotor activity in the mouse, an effect that may have been mediated via the histamine H_1 and/or H_2 receptor.

However, the role of histamine H_3 receptors in stimulant-induced motor activity has not been investigated. We have, therefore, examined the effect of thioperamide and (*R*)-α-methylhistamine on locomotor activity induced by three stimulants which are thought to enhance dopaminergic neurotransmission via different mechanisms. The majority of experiments were performed using amphetamine which releases dopamine from intra-neuronal stores by displacement. Limited studies were also performed with apomorphine, a directly acting dopamine receptor agonist, and the neuronal uptake blocker, cocaine. Parts of this study have been presented, in abstract form, to the British Pharmacological Society (Clapham and Kilpatrick, 1992).

2. Materials and methods

2.1. Experimental protocol

Male CRH mice (18–22 g; Glaxo bred) were placed in individual solid-bottomed activity cages (perspex; 26 × 41 × 16 (high) cm). Each cage was fitted with five infra-red beams on two adjustable levels. The lowest level, level one (set at 30 mm above the cage floor) recorded horizontal motor activity and level two, the higher level (set at 65 mm above the cage floor) recorded vertical activity (e.g. rearing). The computer program used enabled distinction between counts produced by motor activity of the animal and counts produced by tail, head or whisker movements. The former are referred to as slow counts and the latter as

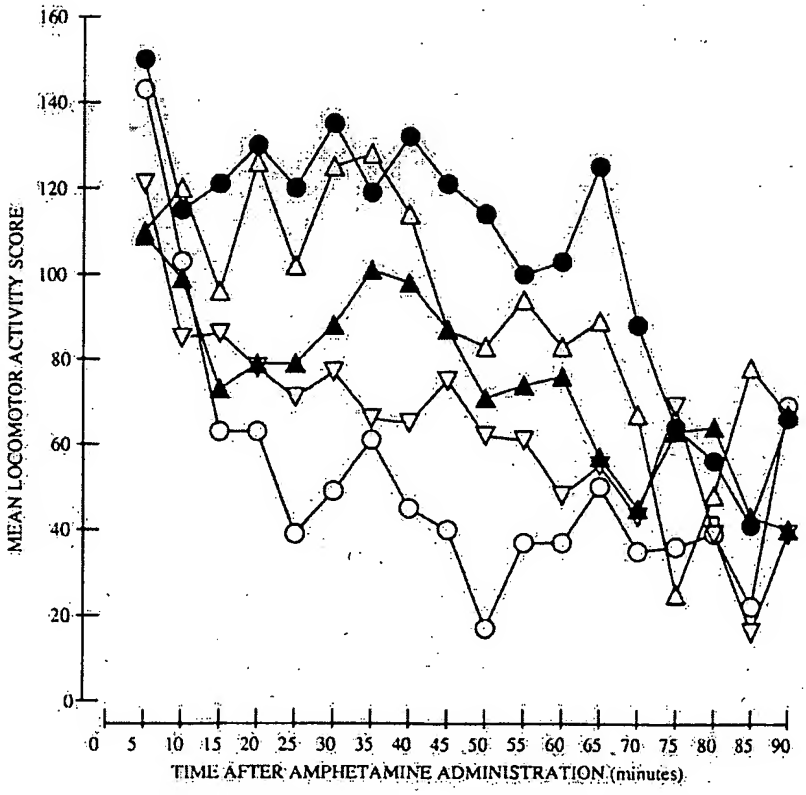

Fig. 1. Effect of the H_3 receptor antagonist thioperamide (THIO; i.p.) on amphetamine (AMPHET; 1 mg · kg^{-1} s.c.)-induced locomotor activity in the mouse. All doses in mg · kg^{-1}; n = 11–12 mice/group. ○ Vehicle, ● AMPHET, △ THIO (0.2) + AMPHET, ▲ THIO (2) + AMPHET, ▽ THIO (10) + AMPHET.

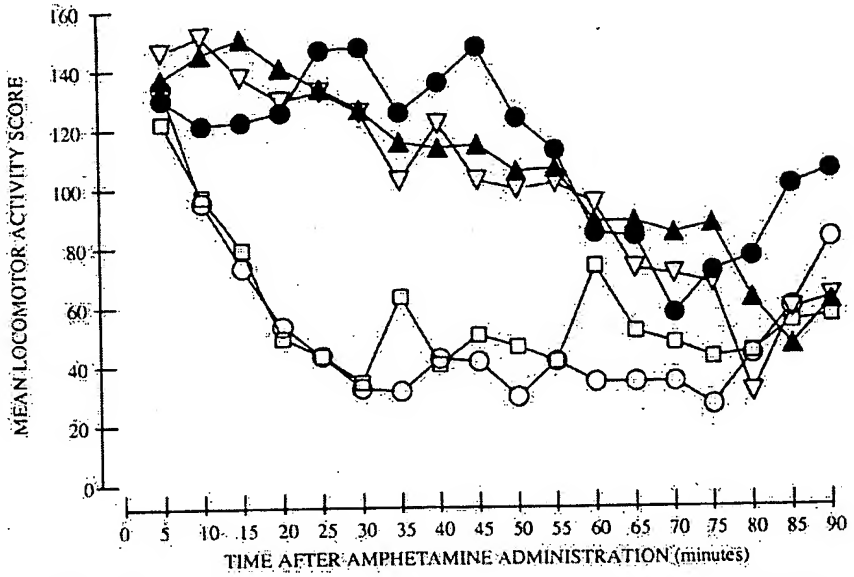

Fig. 2. Effect of the selective H_3 receptor agonist (*R*)-α-methylhistamine (RAMH; i.p.) on amphetamine (AMPHET; 1 mg·kg^{-1} s.c.)-induced and spontaneous locomotor activity in the mouse. All doses in mg·kg^{-1}; n = 22–24 mice/group. ○ Vehicle, ● AMPHET, □ RAMH (20) + Vehicle, ▲ RAMH (3) + AMPHET, ▽ RAMH (20) + AMPHET.

fast counts. Slow counts on the lowest level were used as a measure of horizontal motor activity (locomotor activity) as the animal moved around the cage and interrupted the light beams with its body. Initially, mice were allowed to habituate to the novel environment. Thirty minutes later animals were pretreated with (*R*)-α-methylhistamine or (*R*)-α-methylhistamine vehicle, and thioperamide or thioperamide vehicle intraperitoneally (i.p.; dose volume 0.1 ml per 20 g body weight; each mouse received two injections, one either side of the abdomen). In some studies, 55 min after being placed in the activity cages thioperamide was administered via the intracerebroventricular route (i.c.v.) using an adapted method of that described by Brittain and Handley (1967). A 27 gauge hyperdermic needle (0.32 cm in length) was gently traced across the head from left to right to find the midsagittal suture, which was traced caudally to the coronal suture. The intersection of the midsagittal and coronal sutures is incomplete in the young adult mouse, therefore the point of injection was easily located, the needle inserted perpendicularly and a volume of 5 μl injected using a 10 μl Hamilton syringe. The needle was left in place for 10–15 s before withdrawal to allow the drug time to diffuse into the lateral ventricles. All injection sites were checked for correct placement in the skull and data from mice with misplaced injections were discarded. At 60 min a submaximal stimulant dose of amphetamine (1 mg·kg^{-1}), apomorphine (2 mg·kg^{-1}) or cocaine (5 mg·kg^{-1}) was administered subcutaneously (s.c.; dose volume 0.2 ml per 20 g bodyweight).

Locomotor activity, measured as photocell beam breaks by horizontal motor activity, was recorded every 5 min for a total of 90 min by an online computer (Olivetti M290). Experiments were carried out between

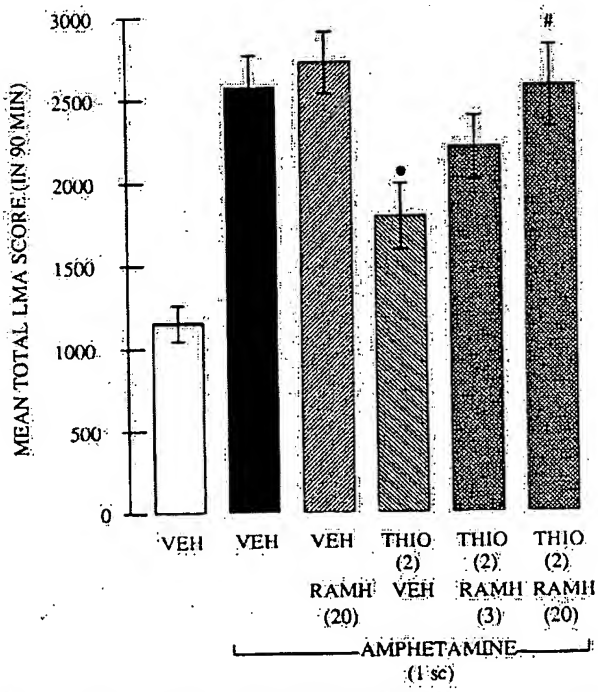

Fig. 3. Effect of (*R*)-α-methylhistamine (RAMH; i.p.) on thioperamide (THIO; i.p.)-induced inhibition of amphetamine (1 mg·kg^{-1} s.c.)-evoked locomotor activity. All doses in mg·kg^{-1}; n = 23–24 mice/group; VEH = water vehicle, * $P < 0.05$ vs. VEH/AMPHETAMINE; # $P < 0.05$ vs. THIO (2)/AMPHETAMINE.

09:00 and 16:00 and drug treatments and activity cages were randomized within each experiment. All drugs were dissolved in distilled water.

2.2. Data analysis

Results are expressed as either the mean locomotor activity score for each 5 min time period following stimulant administration (see graphs) or as the mean total locomotor activity score ± standard error of mean (S.E.M.) over 90 min (see bar graphs). Statistical analysis of the mean total locomotor activity score was performed using analysis of variance followed by either Dunnett's (*) test or Duncan's (#/•) multiple comparison test (* or #/• $P < 0.05$ was considered statistically significant); n = number of mice/drug treatment and the number of experiments performed on separate days is stated where appropriate.

2.3. Drugs

Amphetamine sulphate, apomorphine hydrochloride and cocaine hydrochloride were obtained from Sigma Chemical Company. (*R*)-α-Methylhistamine hydrochloride and thioperamide were synthesized in the Chemical Research Department, Glaxo Research and Development Ltd., Ware.

3. Results

3.1. Effect of thioperamide on amphetamine-induced locomotor activity in the mouse

Thioperamide (0.2, 2 or 10 mg · kg^{-1} i.p.) evoked an inhibition in locomotor activity induced by amphetamine (1 mg · kg^{-1} s.c.) (Fig. 1). The mean total locomotor activity score in 90 min revealed a significant inhibitory effect of the amphetamine response by thioperamide at 2 and 10 mg · kg^{-1} i.p. (vehicle 900 ± 54, amphetamine 1770 ± 97, thioperamide [0.2] + amphetamine 1710 ± 90, thioperamide [2] + amphetamine 1350 ± 74*, thioperamide [10] + amphetamine 1245 ± 69*; * compared with amphetamine alone; data from three experiments; n = 33–36 mice per group; thioperamide dose in square parentheses). Thioperamide at 10 mg · kg^{-1} i.p. inhibited the total locomotor activity score induced by amphetamine by approximately 60%, but at this dose it had no affect on locomotor activity in control animals (mean total locomotor activity score in 90 min 817 ± 75).

3.2. Effect of (R)-α-methylhistamine on amphetamine-induced locomotor activity

(*R*)-α-Methylhistamine (0.3, 3 or 20 mg · kg^{-1} i.p.) had no effect on the increase in locomotor activity

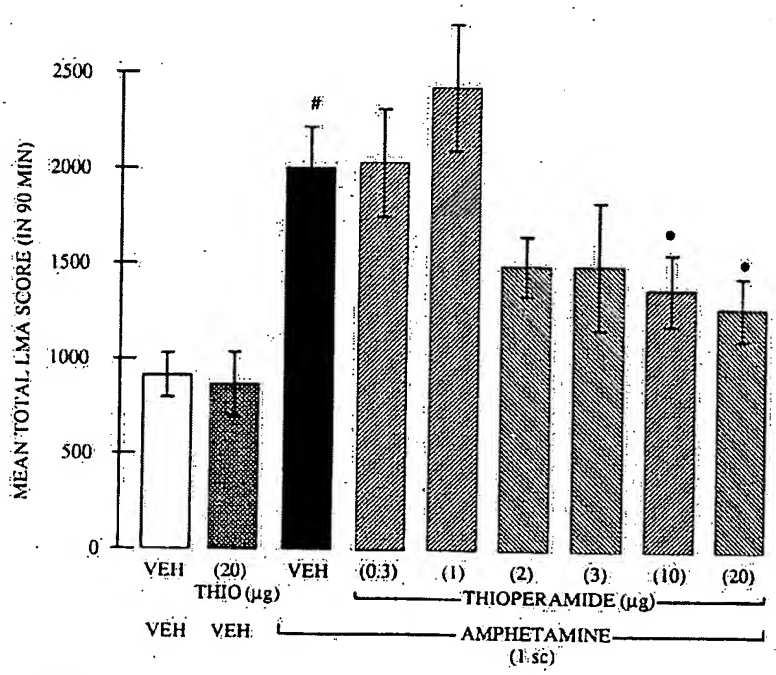

Fig. 4. Effect of thioperamide (THIO; i.c.v.) on amphetamine (1 mg · kg^{-1} s.c.)-induced and spontaneous locomotor activity. THIO doses in μg; n = 9–23 mice/group; VEH = water vehicle, # $P < 0.05$ vs. VEH/VEH; • $P < 0.05$ vs. VEH/AMPHETAMINE.

induced by amphetamine (Fig. 2, 0.3 mg · kg^{-1} dose omitted for clarity). In addition, (*R*)-α-methylhistamine (20 mg · kg^{-1} i.p.) had no effect on locomotor activity in control animals (Fig. 2).

3.3. Effect of (R)-α-methylhistamine on thioperamide-induced inhibition of amphetamine hyperactivity

(*R*)-α-Methylhistamine (20 mg · kg^{-1} i.p.) completely reversed the inhibitory effect of thioperamide (2 mg · kg^{-1} i.p.) on amphetamine (1 mg · kg^{-1} s.c.)-induced hyperactivity; a partial non-significant reversal was also observed at 3 mg · kg^{-1} (*R*)-α-methylhistamine (Fig. 3, mean data from two experiments).

3.4. Effect of thioperamide (i.c.v.) on amphetamine-induced hyperactivity

Central administration of thioperamide (0.3–20 μg i.c.v.; equivalent to 0.015–1 mg · kg^{-1}) evoked a dose-dependent inhibition of amphetamine-induced locomotor activity (Fig. 4, mean data from two experiments). Thioperamide (20 μg i.c.v.) had no effect on spontaneous locomotor activity in control animals (Fig. 4).

3.5. Effect of thioperamide on apomorphine-induced locomotor activity

Thioperamide at 2 or 10 mg · kg^{-1} i.p. evoked a significant inhibition of the locomotor activity response induced by apomorphine (2 mg · kg^{-1} s.c.) (Fig. 5). The mean total locomotor activity scores were vehicle 967 ± 72, apomorphine 1590 ± 59, thioperamide [2] + apomorphine 1083 ± 70*, thioperamide [10] + apomorphine 1350 ± 74*; * compared with apomorphine alone; data from two experiments; $n = 27–30$ mice per group; thioperamide dose in square parentheses. The largest inhibitory effect of approximately 80% was observed at mg · kg^{-1} thioperamide.

3.6. Effect of thioperamide on cocaine-induced locomotor activity

Preliminary studies with cocaine (5 mg · kg^{-1} s.c.) revealed that thioperamide at 2 or 10 mg · kg^{-1} i.p. induced a transient inhibition of cocaine-evoked locomotor activity (Fig. 6). However, when statistical analysis was performed on the total locomotor activity scores,

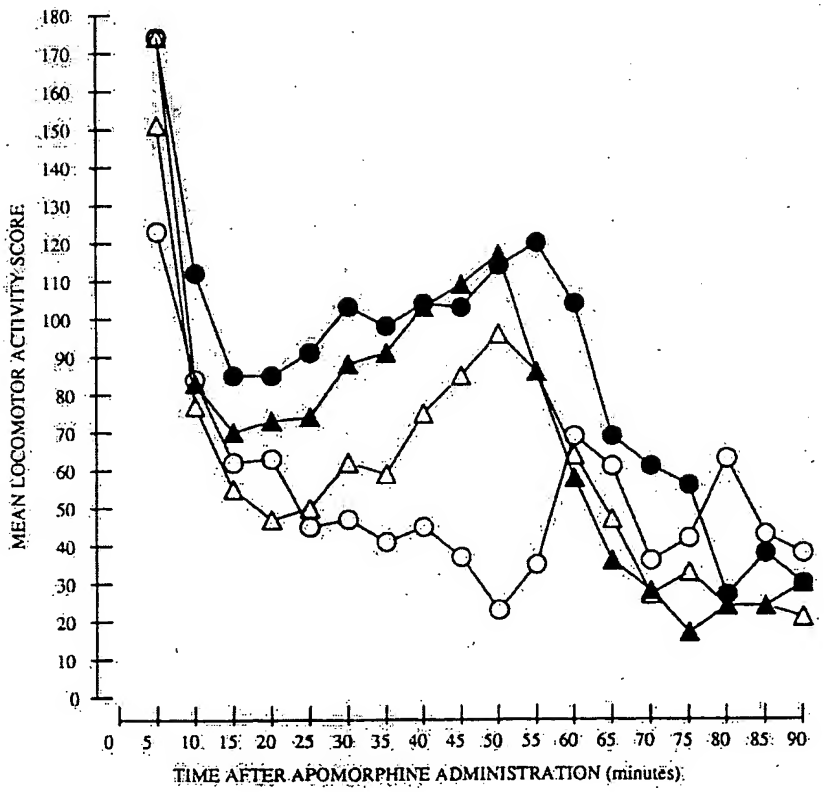

Fig. 5. Effect of thioperamide (THIO; i.p.) on apomorphine (APOMORPH; 2 mg · kg^{-1} s.c.)-induced locomotor activity in the mouse. All doses in mg · kg^{-1}; $n = 9$ mice/group. ○ Vehicle, ● APOMORPH, △ THIO (2) + APOMORPH, ▲ THIO (10) + APOMORPH.

the effect of thioperamide at both doses was not statistically significant (NS) (vehicle 841 ± 117, cocaine 2229 ± 337, thioperamide [2] + cocaine 2234 ± 323 NS, thioperamide [10] + cocaine 3049 ± 232 NS; NS compared with cocaine alone; mean data from 1 experiment; $n = 8/9$ mice per group; thioperamide dose in square parentheses). This lack of effect of thioperamide was influenced by the finding that from the 45 min time point onwards thioperamide at 10 mg · kg^{-1} i.p. appeared to enhance the locomotor activity response to cocaine (Fig. 6).

4. Discussion

In this report we provide the first evidence for a role of the histamine H_3 receptor in modulating stimulant-induced locomotor activity in the mouse. Thus, intraperitoneal administration of the selective H_3 receptor antagonist thioperamide inhibited, in a dose-dependent manner, the increase in locomotor activity induced by amphetamine. Thioperamide at 10 mg · kg^{-1} inhibited the amphetamine response by approximately 60%. Higher doses of thioperamide need to be tested to examine whether a full inhibition can be evoked, as has been observed with dopamine receptor antagonists (Rolinski and Scheel-Krüger, 1973). However, we would predict that thioperamide at 10 mg · kg^{-1} would fully antagonise the histamine H_3 receptor because firstly, drinking studies in the rat revealed that this dose fully attenuated the increase in water consumption evoked by (*R*)-α-methylhistamine (Clapham and Kilpatrick, 1993); secondly, ex vivo binding experiments demonstrated that the same dose of thioperamide, 10 mg · kg^{-1}, inhibited over 80% of specific [^{3}H](*R*)-α-methylhistamine binding to cerebral cortex in the rat and whole brain in the mouse (Taylor et al., 1992).

This inhibitory response evoked by thioperamide was reversed by the selective H_3 receptor agonist, (*R*)-α-methylhistamine. Neither thioperamide nor (*R*)-α-methylhistamine affected spontaneous locomotor activity in their own right indicating that their effects are specific. The effects of thioperamide appeared to be centrally mediated since administration of the H_3 antagonist into the cerebral ventricles, at doses lower than those required systemically, also inhibited amphetamine-induced locomotor activity.

Thioperamide is known to enhance histamine levels in the brain (Arrang et al., 1987; Oishi et al., 1989). Its inhibitory effect on amphetamine hyperactivity in our studies supports the work of Itoh and colleagues (1984)

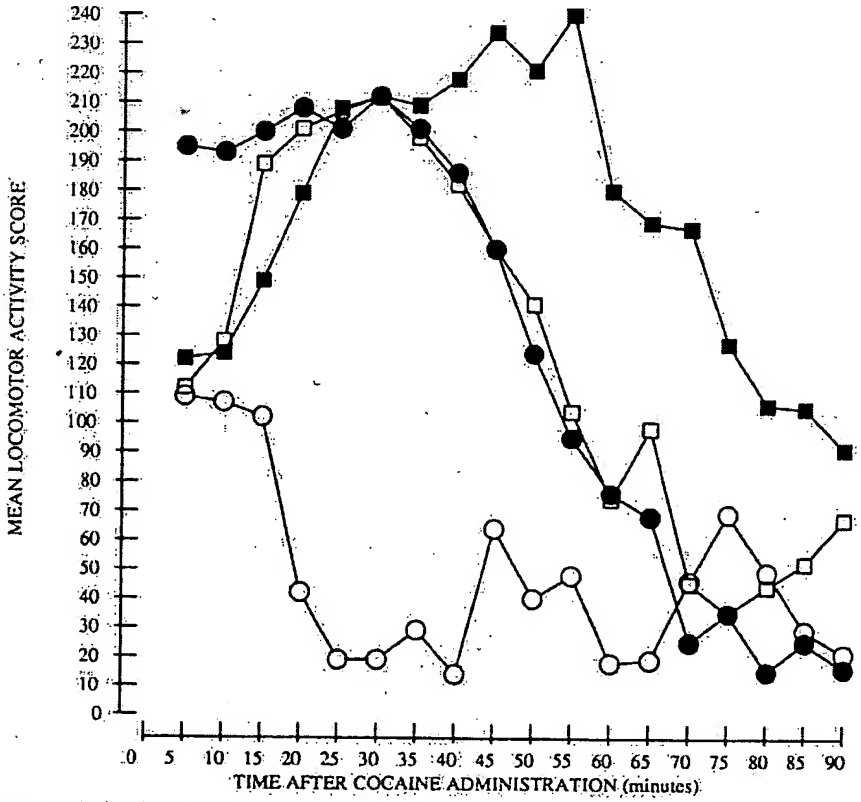

Fig. 6. Effect of thioperamide (THIO; i.p.) on cocaine (5 mg · kg^{-1} s.c.)-induced locomotor activity in the mouse. All doses in mg · kg^{-1}; $n = 8$–9 mice/group. ○ Vehicle, ● COCAINE, □ THIO (2) + COCAINE, ■ THIO (10) + COCAINE.

which provides evidence for an enhancement of histaminergic neuronal function evoking an inhibition of amphetamine-stimulated locomotion. However, histamine H_3 receptors have been shown to modulate not only the release of histamine in the central nervous system, but also acetylcholine (Clapham and Kilpatrick, 1992), 5-hydroxytryptamine (Schlicker et al., 1988), noradrenaline (Schlicker et al., 1989), and, most recently, dopamine (Schlicker et al., 1993). Therefore, care should be exercised when postulating that these effects are mediated solely via modulating histamine levels. The inhibitory effect of thioperamide, clearly indicates that there must be a level of endogenous stimulation of H_3 receptors during amphetamine-induced hyperactivity in the mouse. This reveals an apparent paradox since stimulation or blockade of the H_3 receptor in the control animal had no effect on locomotor activity. The mechanism behind this is not clear, but it may be that the stimulatory effects of amphetamine are mediated through several systems that act together. Blockade of one system may have marked effects, as was observed with those animals treated with thioperamide, whereas stimulation of this system alone e.g. with (*R*)-α-methylhistamine, may be insufficient to cause hyperlocomotion.

The distribution of central H_3 receptors points to a potential involvement in dopamine function. Thus, in autoradiographic studies performed with [^{3}H](*R*)-α-methylhistamine, H_3 receptors were found to be most dense in telencephalic areas including regions with dopaminergic innervation such as the striatum and substantia nigra (Arrang et al., 1987). Dopamine transmission in these areas is known to be involved in the control of movement. Thioperamide may therefore mediate its effects in such brain areas, particularly in light of the recent observation that H_3 receptors play a role in the regulation of dopamine release from mouse striatum. However, stimulation of the H_3 receptor with (*R*)-α-methylhistamine evoked only a small (approximately 15%) reduction in electrically-induced dopamine release, an effect which was reversed by thioperamide (Schlicker et al., 1993). Whether antagonism of the H_3 receptor evokes an effect in its own right was not discussed. Thus, the small modulatory influence of H_3 receptors on dopamine release may not explain our observations. In 1977, Subramanian and Mulder reported that tyramine-induced release of tritiated catecholamines, including dopamine, from slices of striatum was inhibited by preincubation with histamine. Thus, histamine may modulate pre-synaptic catecholamine processes in the central nervous system by causing depletion of nerve terminal transmitter stores. It is, therefore, possible that pretreatment with thioperamide, thereby raising brain histamine levels, causes depletion of dopamine stores and makes those dopamine neurons involved in mediating locomotor activity resistant to the dopamine releasing action of amphetamine. This results in attenuation of the amphetamine-induced locomotor activity response. However, thioperamide also inhibited the hyperactivity induced by the direct dopamine receptor agonist, apomorphine; this suggests that the effects of the H_3 receptor are at a level beyond dopamine neurones. The antagonism of apomorphine hyperlocomotion by thioperamide was not dose-related, 2 mg/kg being more effective than 10 mg/kg. The reasons for this are not clear. In contrast to its inhibitory effect on amphetamine and apomorphine hyperactivity, preliminary studies revealed that thioperamide induced only a small transient reduction in the locomotor activity response evoked by cocaine. At approximately 45 min following cocaine administration, thioperamide (10 mg kg^{-1} i.p.) evoked an increase in cocaine-induced locomotor activity. It is not clear why the locomotor activity-stimulant effects of amphetamine and apomorphine were so sensitive to H_3 receptor antagonism and those of cocaine were not. However, the magnitude of effect induced by cocaine was larger than that induced by amphetamine or apomorphine and therefore, may be more difficult to inhibit. Furthermore, cocaine is less selective for dopamine systems than the other stimulants, preventing the reuptake of a number of neurotransmitters (Farnebo and Hamberger, 1971). Alternatively there may be some metabolic interaction by which thioperamide reduces the metabolism of cocaine, thus negating any reduction in hyperactivity by maintaining circulating levels of the stimulant. Clearly further studies are required and examination of the more selective dopamine uptake blocker, GBR 12909 (Andersen, 1989), may reveal the precise mechanisms involved. In addition, the effect of thioperamide on amphetamine- and apomorphine-induced stereotyped behaviour needs to be addressed. Although our data indicated that thioperamide had no effect on rapid repetitive movements measured as fast counts on both the high and low infra-red beam levels (results not shown), further observation experiments and activity studies are necessary to clarify this point.

In conclusion, we have shown that histamine H_3 receptors may be important in the hyperactivity response induced by certain stimulants. Further study is required before the precise mechanism of this action can be identified.

Acknowledgements

We thank Joanne England for expert technical assistance and Sue Cook for typing this manuscript.

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Antidepressant-like effects of endogenous histamine and of two histamine H_1 receptor agonists in the mouse forced swim test

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1 Effects of substances which are able to alter brain histamine levels and two histamine H_1 receptor agonists were investigated in mice by means of an animal model of depression, the forced swim test.

2 Imipramine (10 and 30 mg kg^{-1}, i.p.) and amitriptyline (5 and 15 mg kg^{-1}, i.p.) were used as positive controls. Their effects were not affected by pretreatment with the histamine H_3 receptor agonist, (R)-α-methylhistamine, at a dose (10 mg kg^{-1}, i.p.) which did not modify the cumulative time of immobility.

3 The histamine H_3 receptor antagonist, thioperamide (2-20 mg kg^{-1}, s.c.), showed an antidepressant-like effect, with a maximum at the dose of 5 mg kg^{-1}, which was completely prevented by (R)-α-methylhistamine.

4 The histamine-*N*-methyltransferase inhibitor, metoprine (2-20 mg kg^{-1}, s.c.), was effective with an ED_{50} of 4.02 (2.71-5.96) mg kg^{-1}; its effect was prevented by (R)-α-methylhistamine.

5 The histamine precursor, L-histidine (100-1000 mg kg^{-1}, i.p.), dose-dependently decreased the time of immobility [ED_{30} 587 (499-712) mg kg^{-1}]. The effect of 500 mg kg^{-1} L-histidine was completely prevented by the selective histidine decarboxylase inhibitor, (S)-α-fluoromethylhistidine (50 mg kg^{-1}, i.p.), administered 15 h before.

6 The highly selective histamine H_1 receptor agonist, 2-(3-trifluoromethylphenyl)histamine (0.3-6.5 μg per mouse, i.c.v.), and the better known H_1 agonist, 2-thiazolylethylamine (0.1-1 μg per mouse, i.c.v.), were both dose-dependently effective in decreasing the time of immobility [ED_{50} 3.6 (1.53-8.48) and 1.34 (0.084-21.5) μg per mouse, respectively].

7 None of the substances tested affected mouse performance in the rota rod test at the doses used in the forced swim test.

8 It was concluded that endogenous histamine reduces the time of immobility in this test, suggesting an antidepressant-like effect, via activation of H_1 receptors.

Keywords: Forced swim test; depression; L-histidine; (S)-α-fluoromethylhistidine; (R)-α-methylhistamine; thioperamide; metoprine; histamine H_1 receptors; 2-(3-trifluoromethylphenyl)histamine; 2-thiazolyethylamine

Introduction

In the aetiology of affective disorders in general, and more specifically in depression, a dysfunction of the monoaminergic (5-hydroxytryptamine, noradrenaline and dopamine) neuronal systems is commonly accepted. Nevertheless, about 7% of treated patients remains chronically depressed even after ten years of therapy (Esposito & Liguori, 1996). Despite another monoaminergic system, the histaminergic system, has been anatomically and functionally defined (Panula *et al.*, 1989; Wada *et al.*, 1991), its role in the control of depressive states has been poorly studied up to now. Papers have been published on the effects of histamine H_1 and H_2 receptor antagonists in various animals models of depression since the 60s (Horovitz *et al.*, 1966; Barnett *et al.*, 1969a,b; Wallach & Hedley, 1979; Onodera & Ogura, 1984; Delini-Stula *et al.*, 1988; Noguchi *et al.*, 1992; O'Neill & Gertner, 1986), often regarded as false positives in these tests (Barnett *et al.*, 1969a,b; Katz & Sibel, 1982; Willner, 1984; Borsini & Meli, 1988; Sunal *et al.*, 1994), while the effect of relatively high doses of histamine itself in the forced swim test has been investigated in only one study (Nath *et al.*, 1988).

In the present work, a different approach to the study of the possible implication of the histaminergic system in depressive-like states was adopted, by investigating the effects of endogenous brain histamine in the mouse forced swim (Porsolt) test. Different substances such as the histamine precursor, L-histidine, the histidine decarboxylase [EC 4.1.1.22] (HDC) inhibitor, (S)-α-fluoromethylhistidine (FMH) (Kollonitsch *et al.*, 1978) and the histamine-*N*-methyltransferase [EC 2.1.1.8] (HMT) inhibitor, metoprine (Duch *et al.*, 1978), have been described as able to alter selectively histamine brain levels. Similarly, the selective histamine H_3 receptor agonist, (R)-α-methylhistamine (RAMH), and antagonist, thioperamide, have been shown to inhibit and stimulate histamine release and synthesis, respectively (Arrang *et al.*, 1987; Garbarg *et al.*, 1989). Therefore, all these substances were used in the present study. To investigate further this topic we thought it worthwhile to test also the two most selective H_1 receptor agonists, 2-(3-trifluoromethylphenyl)histamine (FMPH) (Leschke *et al.*, 1995) and 2-thiazolylethylamine (2-TEA) (Ganellin, 1982).

Preliminary results were presented at the XXVth and XXVIth Annual Meetings of the European Histamine Research Society (Lamberti *et al.*, 1996; 1997).

Methods

Male Swiss albino mice (22-28 g) were used. Twelve mice were housed per cage and fed a standard laboratory diet and

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tap water *ad libitum* during 12 h/12 h light/dark cycles (lights on between 7 h 00 min and 19 h 00 min). The cages were brought into the experimental room the day before the experiment for acclimatization. All experiments were performed between 10 h 00 min and 17 h 00 min.

Forced swim (behavioural despair) test

The forced swim test was performed according to Porsolt *et al.* (1977). The apparatus consisted of two plexiglass cylinders (diameter 10 cm; height 25 cm) vertically placed in a transparent animal cage (21 × 16 × 12 cm) containing 6 cm of water at 22–23°C. Two mice, each of them having received a different treatment, were tested simultaneously by being dropped into the cylinders and left there for 6 min. The duration of immobility was measured with two stop-watches during the last 4 min. A decrease in the duration of immobility is indicative of an antidepressant-like effect.

Rota rod test

The integrity of motor coordination was assessed with a rota rod apparatus (Ugo Basile, Varese, Italy) at a rotating speed of 24 r.p.m., immediately before each swim trial, by counting the number of falls from the rod in 30 s (Vaught *et al.*, 1985).

Drugs

The following drugs were used: amitriptyline HCl (Aldrich), imipramine HCl (Sigma), (R)-α-methylhistamine 2HCl (RBI), thioperamide maleate (RBI), metoprine (Burroughs Wellcome Co), L-histidine HCl (Sigma) and (S)-α-fluoromethylhistidine HCl (FMH; Merck Sharp & Dohme Research Lab.), 2-(3-trifluoromethylphenyl)histamine (Institute of Pharmacy I, Freie Universität Berlin), 2-thiazolylethylamine 2HCl (SmithKline Beecham). All drugs except metoprine were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Metoprine was dissolved in 10% aqueous lactic acid and then diluted with saline (1:30).

Drug concentrations were prepared in such a way that the necessary dose could be injected in a volume of 10 ml kg^{-1} by both s.c. and i.p. routes. For i.c.v. administration a short ether anaesthesia was adopted. Substances were injected in the necessary dose dissolved in 5 μl per mouse according to the method described by Haley & McCormick (1957). To ascertain the exact site of i.c.v. injection, some mice were injected i.c.v. with 5 μl of 1:10 diluted India ink and their brains were examined macroscopically after sectioning.

Statistical analysis

Results are presented as the mean ± s.e. Statistical analysis was performed by means of ANOVA followed by Scheffe's test. P values of less than 0.05 were considered significant. Data were analysed with a computer programme (Number Cruncher Statistical System, Version 5.03 9/92). ED_{50} and ED_{30} values are the doses which produced, respectively, the 50% and 30% of the maximum possible effect, with 95% confidence limits. Both percentages were calculated according to Tallarida & Murray (1984).

Results

Imipramine (10 and 30 mg kg^{-1}, i.p.) and amitriptyline (5 and 15 mg kg^{-1}, i.p.) were used as reference molecules. Both drugs, administered (i.p.) 30 min before the test, were dose-dependently effective, and RAMH did not affect the reduced duration of immobility induced by these antidepressants (Figure 1).

Thioperamide, injected (s.c.) 15 min before the test, reduced the cumulative time of immobility, with a maximum effect at a dose of 5 mg kg^{-1} (Figure 2). Mice treated with RAMH, 10 mg kg^{-1} (i.p.), showed no statistically significant differences in the cumulative time of immobility with respect to controls. Conversely, when RAMH was administered 15 min before thioperamide (5 mg kg^{-1}) treatment, it was able to antagonize completely the antidepressant-like effect of the latter.

Similarly to thioperamide, metoprine, administered 30 min before the test, reduced dose-dependently the time of immobility (Figure 3). Such a reduction was statistically significant at all three doses used (2, 7 and 20 mg kg^{-1}, s.c.), with an ED_{50} of 4.02 (2.71–5.96) mg kg^{-1}. When histamine release was inhibited by pretreatment with RAMH, administered 15 min before, the metoprine (2 mg kg^{-1}) effect was completely reversed.

Also L-histidine showed a dose-dependent antidepressant-like effect when administered at 100, 250, 500 and 1000 mg kg^{-1} (i.p.) 2 h before the test, with an EC_{30} of 587 (499–712) mg kg^{-1} (Figure 4). This effect was statistically significant for the two higher doses tested. Mice pretreated with FMH administered at a dose of 50 mg kg^{-1} (i.p.) 4 or 15 h before saline treatment were not significantly different from controls. In contrast, when FMH was administered 4 h before treatment with 500 mg kg^{-1} L-histidine, the effect of the latter was slightly reduced. This reduction was stronger and statistically significant when FMH was administered 15 h before L-histidine (Figure 4).

Due to their inability to cross the blood brain barrier, both FMPH and 2-TEA were administered i.c.v. 15 min before the

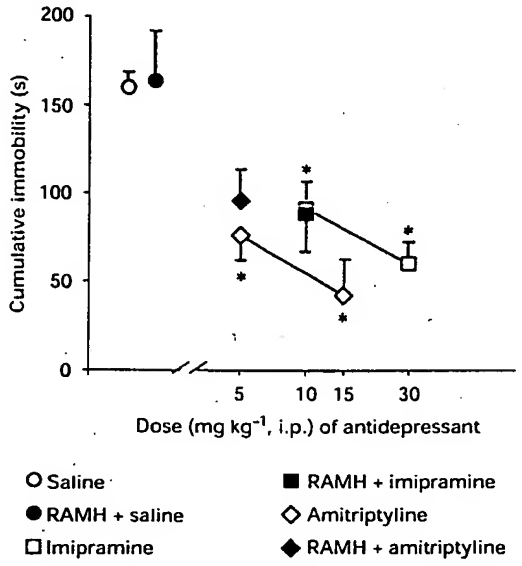

Figure 1 Effects of the tricyclic antidepressants imipramine HCl and amitriptyline HCl alone and in combination with (R)-α-methylhistamine 2HCl (RAMH) in the mouse forced swim test. RAMH (10 mg kg^{-1}, i.p.) was administered 15 min before saline or antidepressant treatment, which were injected i.p. 30 min before the test. *$P<0.001$ versus saline controls (ANOVA followed by Scheffe's test). Each point represents the mean (with s.e.mean shown by vertical lines) of 10–50 mice.

test. Both FMPH and 2-TEA were dose-dependently effective in reducing the time of immobility. FMPH, administered in four different doses (0.3, 1, 2.65 and 6.5 μg per mouse), had a statistically significant effect at the highest dose (Figure 5) and an ED_{50} of 3.6 (1.53–8.48) μg per mouse, while 2-TEA proved to be significantly effective at all three doses tested (0.1, 0.3 and 1 μg per mouse) (Figure 5), with an ED_{50} of 1.34 (0.084–21.5) μg per mouse.

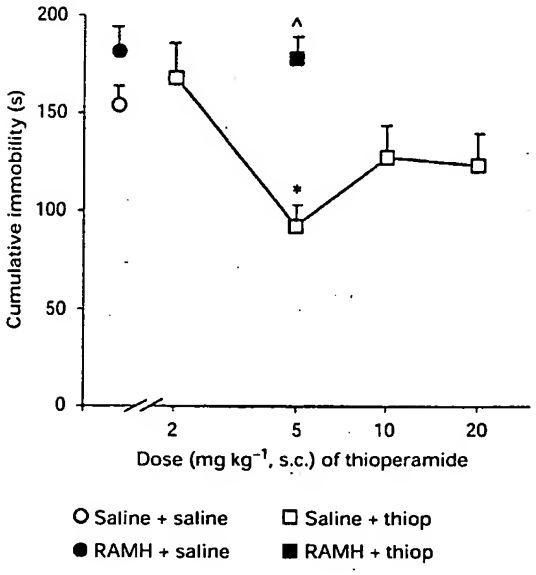

Figure 2 Antidepressant-like effect of thioperamide maleate (Thiop) and its antagonism by (R)-α-methylhistamine 2HCl (RAMH) in the mouse forced swim test. RAMH (10 mg kg^{-1}, i.p.) was administered 15 min before saline or thioperamide treatment, which was injected s.c. 15 min before the test. $^{*}P<0.05$ versus saline controls; $^{\wedge}P<0.001$ versus thioperamide (5 mg kg^{-1})-treated mice (ANOVA followed by Scheffe's test). Each point represents the mean (with s.e.mean shown by vertical lines) of 11–26 mice.

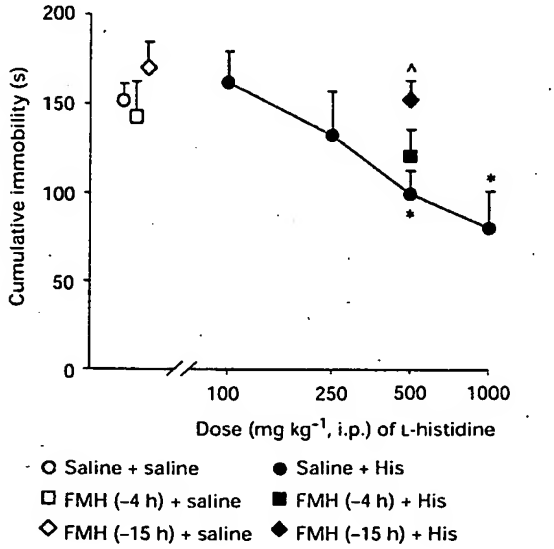

Figure 4 Antidepressant-like effect of L-histidine (His) and its antagonism by (S)-α-fluoromethylhistidine HCl (FMH) in the mouse forced swim test. FMH (50 mg kg^{-1}, i.p.) was administered 4 or 15 h before saline or His treatment, which were injected (i.p.) 2 h before the test. $^{*}P<0.05$ versus saline controls; $^{\wedge}P<0.05$ versus His (500 mg kg^{-1})-treated mice (ANOVA followed by Scheffe's test). Each point represents the mean (with s.e.mean shown by vertical lines) of 10–36 mice.

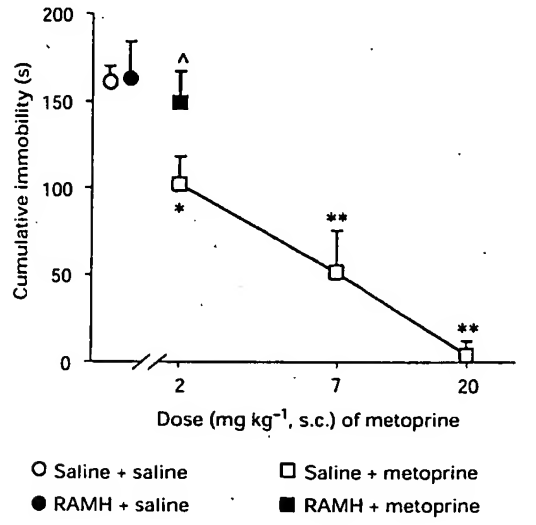

Figure 3 Antidepressant-like effect of metoprine and its antagonism by (R)-α-methylhistamine 2HCl (RAMH) in the mouse forced swim test. RAMH (10 mg kg^{-1}, i.p.) was administered 15 min before saline or metoprine treatment, which were injected s.c. 30 min before the test. $^{*}P<0.05$, $^{**}P<0.001$ versus saline controls; $^{\wedge}P<0.05$ versus metoprine (2 mg kg^{-1})-treated mice (ANOVA followed by Scheffe's test). Each point represents the mean (with s.e.mean shown by vertical lines) of 11–28 mice.

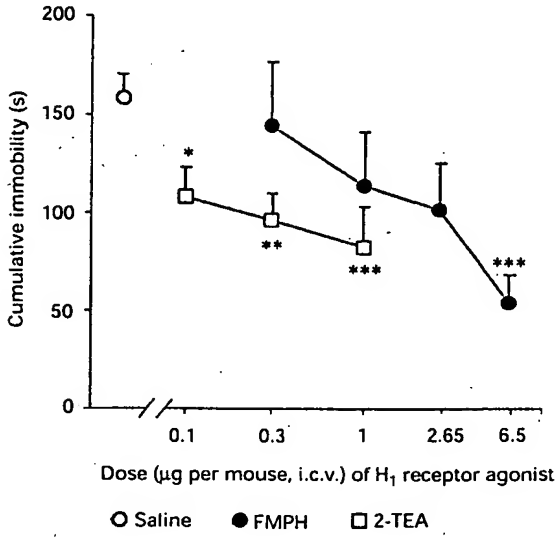

Figure 5 Antidepressant-like effects of 2-(3-trifluoromethylphenyl)-histamine dihydrogenmaleate (FMPH) and 2-thiazolylethylamine 2HCl (2-TEA) in the mouse forced swim test. The test was performed 15 min after treatment with either FMPH or 2-TEA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus saline (i.c.v.-treated) controls (ANOVA followed by Scheffe's test). Each point represents the mean (with s.e.mean shown by vertical lines) of 10–32 mice.

In the rota rod test none of the substances at the doses used altered mouse performance in comparison to one group of 16 controls, which scored 0.47 ± 0.19 falls from the rod in 30 s.

Discussion

The present results suggest an involvement of the histaminergic system in the modulation of depressive states as evidenced by the forced swim test. In fact, substances able to enhance histaminergic transmission reduced the duration of immobility, indicating a possible antidepressant effect. This antidepressant-like effect might be due to activation of histamine H_1 receptors, since two selective H_1 agonists caused reduced immobility as well.

The two reference molecules used, the tricyclic antidepressants, imipramine and amitriptyline, were tested as positive controls in our condition. Thioperamide showed a statistically significant antidepressant-like effect at a dose of 5 mg kg^{-1}. Since thioperamide was described as a selective antagonist for histamine H_3 presynaptic autoreceptors (Arrang *et al.*, 1987), the reduced duration of immobility may be due to enhanced histamine release from the synaptic terminals. The antagonism of thioperamide effect by the H_3 receptor agonist, RAMH, administered at a dose ineffective *per se* on the duration of immobility, is not likely to be aspecific, since RAMH did not affect the behaviour of mice treated with the classical antidepressants. Furthermore, studies have demonstrated that several antidepressant drugs, including amitriptyline, exhibit low or negligible affinities for the histamine H_3 receptor (Kathmann *et al.*, 1994).

Interestingly, at higher thioperamide doses, 10 and 20 mg kg^{-1}, a less pronounced effect was observed. Since thioperamide has been shown to have a K_i of 4 nM on the H_3 and >10,000 nM on the H_1 or H_2 receptors (Schwartz *et al.*, 1990), the hypothesis of a postsynaptic antagonism can be ruled out. Instead, the less pronounced effect is probably due to competition between thioperamide and the endogenous histamine released on the H_3 receptor. Such a partial reversal of thioperamide effect with increasing doses was also observed while tests were being performed for antinociception (Malmberg-Aiello *et al.*, 1994) and locomotor activity (Sakai *et al.*, 1991).

A further approach to study the effects of endogenous histamine was to inhibit its catabolism or enhance its synthesis. The dose-dependence of the decrease in immobility induced by the HMT inhibitor, metoprine, reflects the degree of inhibition of the enzyme, since, as demonstrated by Hough *et al.* (1986), 5 mg kg^{-1} (i.p.) metoprine reduced whole brain HMT activity by 70%, while a 90% reduction could be obtained with 20–30 mg kg^{-1} (i.p.). The complete antagonism of such a decrease in immobility by pretreatment with RAMH, due to inhibition of histamine release, demonstrates that metoprine activity is due to released histamine which has not been catabolized.

Histamine synthesis can be enhanced by systemic administration of the precursor, L-histidine, since the enzyme HDC is not saturated in physiological conditions (Schwartz *et al.*, 1972; Abou *et al.*, 1973). Furthermore, histidine loads (1000 mg kg^{-1}) in mice produce a rise in histamine whole brain levels with a maximum occurring 1 h after treatment, and a slowly decreasing plateau thereafter (Taylor & Snyder, 1972). In our experience, antinociception following administration of 50 or 1000 mg kg^{-1} (i.p.) reached a maximum effect 2 h after treatment in the mouse abdominal constriction test (Malmberg-Aiello *et al.*, 1994). Therefore, in the present study we treated mice with L-histidine 2 h before the test. To demonstrate that L-histidine activity was actually due to its conversion into histamine, mice were also pretreated with FMH, a highly selective irreversible HDC inhibitor which has been demonstrated to halve brain histamine levels in mice within 2 h up to 48 h, at the dose of 25 mg kg^{-1} (i.p.) (Maeyama *et al.*, 1982). Antagonism by FMH was complete when the substance was administered 15 h before L-histidine.

Thus, in the present study three approaches were adopted to investigate the effects of endogenous histamine, and the results obtained with all of them suggest a possible positive control by the histaminergic system in depressive-like states. A further indication for the involvement of the histaminergic system in the control of depressive states is given by the effects of electroconvulsive shocks (ECS). ECS was shown to reduce the time of immobility in the rat forced swim test (Porsolt *et al.*, 1978), and at present is one of the therapeutic strategies adopted in some cases of depression, such as when patients respond poorly to medication (Weiner & Krystal, 1994). It has been demonstrated that histamine levels in rat cerebral cortex are significantly decreased 24 h after a single ECS, indicating that histamine release had occurred, while HDC activity was increased both 24 h after a single shock and 1 and 24 h after repeated ECS (Zawilska & Nowak, 1985). All this seems to be in contrast with the work by Nath *et al.* (1988), who found an increase in the duration of immobility in the mouse forced swim test following intracerebral administration of histamine. On the other hand, the histamine doses at which such an increase was observed (50, 100 and 200 μg per mouse) were rather high, since 100 μg per mouse (i.c.v) were shown to induce facial convulsions (Malmberg-Aiello *et al.*, 1994), and 200–400 μg per rat to produce catalepsy (Glick & Crane, 1978). For this reason, all the substances used in the present work were also tested by means of a rota rod apparatus before the forced swimming test was performed, to make sure that they did not influence the normal motor activity of the mice. Since ataxic mice are not able to coordinate movements and fall from the rotating rod, while excited animals tend to jump off the rod, the good performance on the rod by mice in the present study indicates the results obtained with the forced swim test are not due to altered motor activity induced by the substances at the doses used. Furthermore, drugs which have a known psychostimulant effect, like (+)-amphetamine and caffeine, at the same doses at which they are able to decrease the time of immobility in the rat forced swim test, also show a significantly increased motor activity in an open field (Porsolt *et al.*, 1978).

After the effects of endogenous histamine had been studied, it seemed worthwhile to investigate the consequences of H_1 receptor activation. This was possible thanks to the recent development of a potent and selective H_1 receptor agonist, FMPH. The molecule is 2138 fold more selective on H_1 than on H_2 receptors, and discriminates between H_1 and other receptors by >64 (H_1:H_3), 1000 (H_1:M_3), 105 (H_1:α_1), 708 (H_1:β_1) and 71 (H_1:5-HT_{2A}) (Leschke *et al.*, 1995). For comparison, the better known H_1 receptor agonist, 2-TEA, was studied, although it does not discriminate between H_1 and H_2 receptors on a wide range, its relative potency on H_1:H_2 receptors being about 12:1 (Ganellin, 1982). Both FMPH and 2-TEA were administered in doses which completely prevented the antinociception induced by the H_1-antagonist, pyrilamine, in our nociception tests (Malmberg-Aiello *et al.*, unpublished observation). The dose-dependent efficacy of the two H_1 agonists in reducing the time of immobility strongly suggests a role for the H_1 receptor in mediating the responses of the mice in the forced swim test following histaminergic system activation. It is interesting to note that the two substances were

equipotent in producing their effects, considering that FMPH has a 2.5 fold higher molecular weight than 2-TEA, and the same 2.5 fold difference was evident when their ED_{50} values were compared.

The antidepressant-like effect of H_1 receptor agonists is not surprising. In fact, FMPH was shown to increase waking in rats (Monti *et al.*, 1994), and the role of the histaminergic system in waking states has been variously demonstrated (Kalivas, 1982; Monti, 1993; Lin *et al.*, 1994). On the other hand, the antidepressant effect of a one-night sleep deprivation, which produces an immediate and short-lasting therapeutic effect in about 60% of depressed patients, has also been described (Gillin, 1983). Therefore, it can be speculated that the therapeutic effect of sleep deprivation, the waking role of the histaminergic system (via H_1 receptors) and the antidepressant-like effects evidenced in the present study are somehow related to each other. Furthermore, the immediate beneficial effect of sleep deprivation might also explain the effects observed with acute administration of the substances used in the present study.

In conclusion, the present work suggests a possible importance of the histaminergic system in the control of depressive states, as indicated by the positive role of endogenous histamine in the forced swim test. A role for H_1 receptors has been defined by the use of H_1 receptor agonists, while the eventual implication of H_2 receptors still remains to be thoroughly investigated, mainly due to the lack of really selective H_2 agonists. It is speculated that a further study on the central histaminergic system might provide new therapeutic possibilities for the treatment of depression.

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Histaminergic Ligands Attenuate Barrel Rotation in Rats Following Unilateral Labyrinthectomy

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SUMMARY

In this paper we present a unilateral labyrinthectomy (UL) surgical procedure in rats that was derived from previous techniques. The utility of this model to assess vestibular dysfunction was evaluated by examining the ability of clinically used histaminergic agents and more selective H_3 receptor antagonists to attenuate of UL-induced body rotations. Unilateral labyrinthectomy was performed by injection of ethanol into the rat right inner ear. An elevated body rotation test (EBRT) was used to assess the abnormal rotational behavior induced by UL. Scores of "3" to "0" were used to characterize the degree of abnormal behavior according to the latency of body rotations to begin. Our results demonstrate that: i) 100 µl ethanol induced robust behavioral changes, which was used in further experiments; ii) the clinically used antivertigo agent, astemizole, significantly reduced the rotational behavior in UL rats; iii) the more potent H_3 antagonists, thioperamide and GT-2016, were more efficacious than betahistine, a mixed H_3 antagonist and H_1 agonist. These results indicate that this model may be a potential tool for testing novel drugs for antivertigo effects and provide better support to the role of the histaminergic system in the control of vestibular function.

Key words: Vertigo - Unilateral labyrinthectomy - Barrel rotation - Betahistine - Astemizole - Thioperamide - GT-2016

INTRODUCTION

Vertigo may develop in association with many diseases which originate within the central nervous system or in the periphery. Unfortunately, the majority of patients who suffer from vertigo cannot be treated from an etiological aspect, as the otological or neurological origins of those diseases are not fully understood (1, 2). In these cases, treating the symptoms of vertigo has been a primary goal of clinical practice (2). To meet clinical needs, a broad range of antivertigo drugs is available on the market, but their effectiveness has been unsatisfactory (1-3).

One of the reasons for the partial effectiveness of most antivertigo drugs may be the serendipitous nature of the discovery of their antivertigo effects at the clinical level. Despite the large unmet medical need for antivertigo drugs, few animal models of vertigo exist, hindering the drug-discovery process. Thus, a reliable *in vivo* model of vestibular dysfunction would be desirable. One potential approach would be to study the behavioral recovery from a vestibular lesion. This approach would help to bridge the gap between the advances in the understanding of the basic anatomy and physiology of the vestibular system with the more speculative and experimental use of drugs affecting the vestibular system.

Based on the knowledge that vestibular dysfunction is the primary cause of vertigo (1),

one experimental strategy that has been used for the *in vivo* study of vertigo is an animal model in which the primary vestibular input has been unilaterally destroyed (unilateral labyrinthectomy, UL). UL can be achieved in two ways. One approach is to surgically expose the bony labyrinth and remove the inner tissue via suction or to destroy the membrane labyrinth with an application of ethanol or selective ototoxic chemicals like streptomycin directly on the labyrinth membrane (4-7). This procedure can be augmented by cutting the vestibular nerve and/or removing Scarpa's ganglion (5, 6). A second approach is to place a known amount of an ototoxic compound into the middle ear (8).

In the present paper, we describe a modified UL procedure in which ethanol was injected directly through the round window into the inner ear of rats. To evaluate the model, we assessed the behavioral effects of UL, and the effects of histaminergic agents presently used in the clinic to treat vertigo. We also explored the potential of this model to discover more effective drugs to treat vertigo.

MATERIALS AND METHODS

Animals

Adult male Long Evans rats weighing 250-350 g (Harlan Sprague-Dawley, Inc. Indianapolis, IN, USA) were used in all experiments. Rats were housed individually in a climate-controlled environment on a 12-hour light/dark cycle.

Surgical procedure for unilateral labyrinthectomy

All animals received a right side labyrinthectomy using protocols approved by IACUC. Briefly, each rat under halothane anesthesia was placed on a plate with its left side facing down and its abdomen facing the surgeon. Under a dissection microscope, a 30½ gauge needle connected to a small syringe filled with 100% ethanol was passed through the right external ear canal and tympanic membrane. The needle was then inserted into the round window. Lymph-fluids which leaked from the injection site were aspirated out by a thin glass transfer pipet-tip. A volume of 100 µl ethanol was injected into the round window, followed by insertion of a small piece (approximately 0.2 x 0.2 cm) of gelfoam moistened with 100% ethanol into the right middle ear. Rats were placed back into their cage with their left side facing down. In control rats, the inner ear was injected with an equal volume of saline. Preliminary experiments evaluated the effects of 50 µl ethanol.

Monitoring of behavioral changes in UL rats

Behavioral changes in UL rats were assessed at 2, 3, 4, 5, and 6 h after ethanol or saline injection. The elevated body-rotation test (EBRT), which was developed in our laboratory and described previously (9), was chosen to measure the characteristic asymmetrical motor behavior. Briefly, the rat was elevated 2-3 cm above the bench top for 30 seconds by holding its tail. The severity of the rotations was scored using the EBRT which was determined by the latency for the rat to start rotating: rotation initiated within the first 10 seconds (score of 3), within the second 10 seconds (score of 2), and in the last 10 seconds (score of 1), with a score of zero assigned to rats that did not rotate. Ethanol treated rats that received a score of 3 in the first 2 hours after UL were used for further investigation. These rats were treated intraperitoneally (i.p.) with vehicle or the test compound 2 hours after ethanol injection, and the EBRT was carried out every half hour up to 4 hours following drug administration. Two EBRT scores were recorded for each rat within each 1-hour period; the mean of these two scores represented the turning behavior of rats during this time period.

Drugs

The effects of astemizole, betahistine (Sigma Chemical Company, St. Louis, MO, USA), thioperamide (Ikesearch Biochemical International, Natick, NM, USA), and GT-2016 (synthesized at Abbott Laboratories) on

UL-induced rotation in rats were investigated in four separate experiments. Astemizole, and GT-2016 were prepared in a vehicle of 2% Tween in saline. Betahistine and thioperamide were prepared in physiological saline solution.

Experiment 1: 32 rats were divided into 4 groups: vehicle (n = 7), astemizole 12.5 (n = 9), 25 (n = 9), or 50 μmol/kg (n = 7). Experiment 2: 57 rats were divided into 5 groups: saline (n = 13), betahistine 14 (n = 7), 36 (n = 8), 71 (n = 17), or 143 μmol/kg (n = 12). Experiment 3: 53 rats were treated with: saline (n = 17), thioperamide 2.4 (n = 13), 7.3 (n = 13), or 24 μmol/kg (n = 10). Experiment 4: 42 rats were divided into 3 groups: vehicle (n = 12), GT-2016 24 (n = 16), or 47 μmol/kg (n = 14).

Data analysis

The mean EBRT score was calculated for each group of rats for each 1-hour interval. Data were analyzed by the Mann-Whitney *U*-test, with a significant effect considered at $p < 0.05$.

RESULTS

All UL rats showed asymmetrical behaviors (head tilt, nystagmus and barrel rotation) after recovering from anesthesia. In the following experiments, in addition to the observation of their nystagmus and head tilt, the EBRT was used to assess the asymmetrical behavioral changes.

Behavioral effects of ethanol injection into the inner ear

All rats which underwent a right side labyrinthectomy rolled toward the ethanol injected side along their longitudinal axis following recovery from halothane anesthesia, then rested in a position with their labyrinthectomized side pressed against the floor of the cage after a few rotations. They demonstrated a constant 360° turning behavior along the longitudinal axis when suspended by the end of the tail a few centimeters above the bench top. These rats generally showed severe rotations which lasted for several days (Fig. 1A). The control rats, which lost lymph-fluids and received 100 μl saline in their inner ear, showed

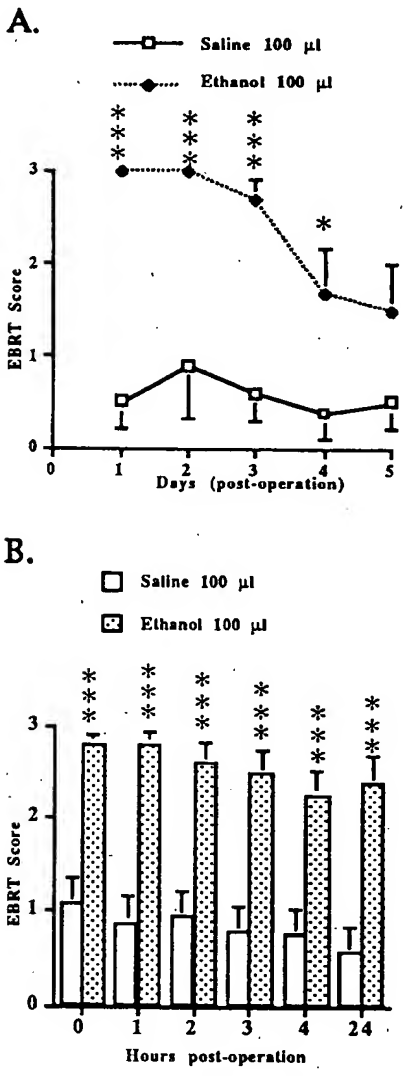

FIG. 1. Effect of ethanol on EBRT scores. A) Ethanol-treated rats (n = 9) maintain significantly greater EBRT scores for up to 4 days post-lesion compared to saline-injected controls (n = 9). B) The EBRT scores are significantly greater in ethanol-injected rats (n = 16) than in saline-injected control ones (n = 16). Data represents the mean scores ± SEM of each group at hourly time points, respectively. $^{*}p < 0.05$, $^{***}p < 0.001$.

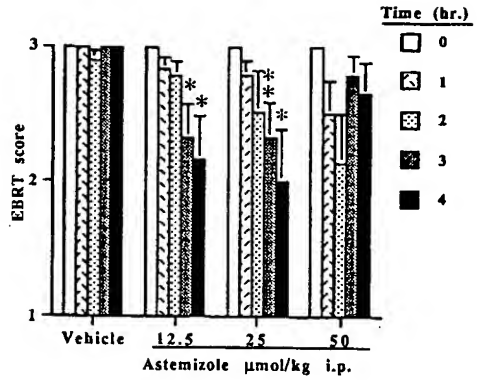

FIG. 2. Effect of astemizole on EBRT scores. Astemizole was administered 2 h post-UL. Each bar represents the mean EBRT scores ± SEM of each group treated with vehicle (2% Tween, n = 7), or astemizole at 12.5 (n = 9), 25 (n = 9), or 50 µmol/kg (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$ by Mann-Whitney *U*-test.

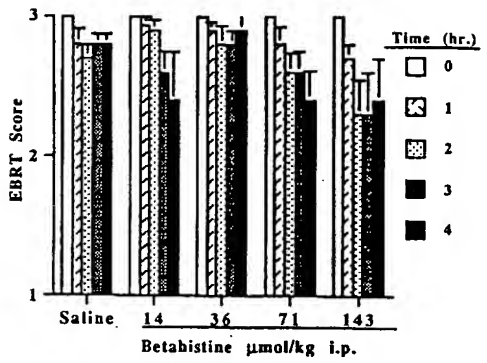

FIG. 3. Effect of betahistine on EBRT scores. Betahistine was administered 2 h post-UL. Each bar represents the mean EBRT scores ± SEM of each group treated with saline (n = 13), or betahistine at 14 (n = 7), 36 (n = 8), 71 (n = 17), or 143 µmol/kg (n = 12).

mild and transient asymmetric behavior. Rotational behavior was significantly reduced in these rats compared to ethanol-treated rats (Fig. 1A and B).

A preliminary experiment showed that rats injected with 100 µl ethanol were more likely to have EBRT scores of 3 than rats receiving 50 µl of ethanol. Eight out of 15 rats injected with 50 µl ethanol failed to reach a score of 3 at 2 h, and the scores varied from 0 to 3 in the subsequent 3-6 h post-injection observation period. In rats treated with 100 µl ethanol, 9 out of 12 rats had scores of 3 at 2 h; among these 9 rats, the EBRT scores were more consistent throughout the assessment period compared to the rats that received 50 µl ethanol. Based on these findings, the 100 µl volume of ethanol was used for all subsequent experiments.

Effect of histaminergic compounds

Astemizole showed a tendency to attenuate rotational behavior in UL rats, with a significant effect at 3 and 4 h after treatment compared to the control group. The effective doses were 12.5 µmol/kg ($p < 0.05$ at 3- and 4-h time-points) and 25 µmol/kg ($p < 0.01$ and $p < 0.05$ at 3- and 4-h time-points), respectively (Fig. 2).

In contrast, the effects of betahistine were more modest. As can be seen in Figure 3, betahistine tended to attenuate the rotational behavior at 71 µmol/kg and 143 µmol/kg.

The effect of thioperamide on barrel rotation is shown in Figure 4. The ability of thioperamide to attenuate barrel rotations was apparent within the

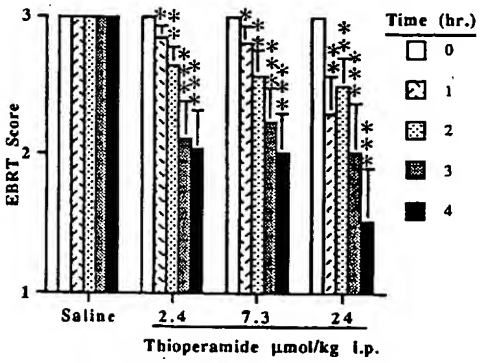

FIG. 4. Effect of thioperamide on EBRT scores. Thioperamide was administered 2 h post-UL. Each bar represents the mean EBRT scores ± SEM of each group treated with saline (n = 17), or thioperamide at 2.4 (n = 13), 7.3 (n = 13), or 24 µmol/kg (n = 10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by Mann-Whitney *U*-test.

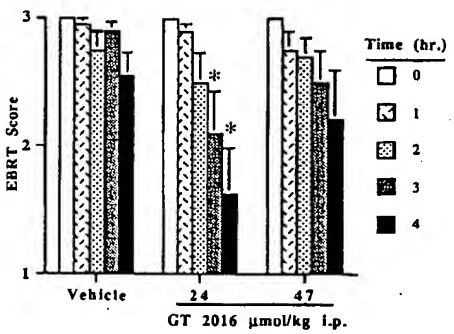

FIG. 5. Effect of GT-2016 on EBRT scores. GT-2016 was administered 2 h post-UL. Each bar represents the mean EBRT scores ± SEM of each group treated with vehicle (2% Tween, n = 12), or GT-2016 at 24 (n = 16), or 47 µmol/kg (n = 14). *$p < 0.05$ by Mann-Whitney *U*-test.

first hour following treatment of 2.4 µmol/kg ($p < 0.05$), 7.3 µmol/kg ($p < 0.05$), and 24 µmol/kg ($p < 0.01$). The significant effect of these doses increased over the remainder of the observation period to $p < 0.01$ at 2 h and $p < 0.001$ at 3 and 4 h post-treatment.

Figure 5 shows that GT-2016 attenuated rotational behavior in UL rats. A dose of 24 µmol/kg, but not 47 µmol/kg, significantly reduced the EBRT scores at 3 and 4 h ($p < 0.05$) post-treatment.

DISCUSSION

In our study, we present a simplified procedure to induce UL in which unilateral injection of 100 µl ethanol into the round window in rats induced severe asymmetrical behavioral changes. Typical behaviors included nystagmus, head tilt, and barrel rotation. This behavioral pattern was similar to behaviors seen in rats in which one side of the membrane labyrinth was exposed and destroyed by ethanol (4). Although the bony labyrinth was not opened in the present study, the outflow of lymph fluid was an obvious sign that the tip of the needle was inserted into the semicircular canal.

In saline-injected controls, in which there was ipsilateral loss of lymph-fluid, the vestibular nuclei presumably received unequal afferent inputs from the intact labyrinth and the saline-injected one. For this reason, the control rats also showed some barrel rotation and turned toward the labyrinthectomized side to a position with this side of the body against the cage floor immediately upon recovering from anesthesia. These control rats showed transient asymmetrical behaviors similar to the ethanol-treated rats; however, the control rats' abnormal behaviors were much less severe and were of shorter duration (Fig. 1). In contrast, ethanol-treated rats not only lost lymph-fluid from the labyrinth, but were presumably also subjected to neuronal damage, which increased the severity and duration of the asymmetrical behaviors (Fig. 1).

This effect of ethanol was demonstrated in our initial study in which the severity of the rotational behavior was rated by the EBRT in rats treated with two different volumes of ethanol (50 µl or 100 µl). Seventy-five percent of rats injected with 100 µl ethanol had scores of 3 at 2 h post-surgery as compared to less than 50% of rats injected with 50 µl ethanol. In the rats injected with 100 µl ethanol, more reliable scores of 3 were maintained over a longer period of time, while scores ranged from 0 to 3 over the same period in the rats injected with 50 µl ethanol.

The finding that a 100 µl ethanol injection provided relatively consistent EBRT scores in rats over the 2- to 6-h post-lesion observation period implies that in this period of time, the process of vestibular compensation was still far from complete. This relatively steady baseline enabled the evaluation of antivertigo compounds. Because most of the 100 µl ethanol-treated rats maintained scores of 3 during this period, a decrease of the scores in drug-treated groups could be attributed to effects of the compounds. The extent to which the EBRT score was decreased provided a benchmark for the effectiveness of the compound to reduce asymmetrical behavior. For example, we observed that the histaminergic compounds, *e.g.*, betahistine and astemizole, which are used clinically to treat vertigo, showed weak to moderate ability to attenuate the turning behavior in our model, thus serving to validate the model.

To dissociate the effect of a compound to attenuate barrel rotation from reducing spontaneous locomotion, activity tests including open-field locomotion and rotorod were carried out in rodents. Any dose of compound that decreased spontaneous activity was excluded from testing in the UL model (data not shown).

Astemizole is a nonsedating antihistaminergic compound that minimally passes the blood brain barrier and has antivertigo properties without sedation (10, 11). A study by Norris and Jackson (12) demonstrated that, following an intraperitoneal injection of radioactive astemizole in adult mice, radioactivity could be detected in the inner ear, indicating that astemizole was transported into the inner ear and/or lymph fluids. In a separate study, the activity of the ampullar nerve was recorded from isolated posterior canals of the frog in a bath of either astemizole or its diluent fluids and revealed that astemizole had inhibitory effects on vestibular nerve activity (12).

These experimental findings help to explain the clinical observation that astemizole only helps patients with dizziness of a peripheral origin (11, 13). Our finding that astemizole decreased the EBRT scores in the unilateral inner ear ethanol-treated rats implies that this model could be used to test compounds that act peripherally.

A unique phenomenon in the pharmacotherapy of vertigo is that both histaminergic agonists and antagonists are utilized clinically. Based on the vasodilatory effect of betahistine, this histaminergic compound was recommended in the 1970s by otolaryngologists for use only in vertigo having neurovascular and vascular etiology but not for general dizziness (14). Following the discoveries of betahistine's penetration into the CNS and its H_3 antagonism (15), the therapeutic action of betahistine has been attributed to its central effect via its H_3 antagonist activity rather than its H_1 agonism (15-18). The mixed pharmacology of betahistine may clarify the present data in which betahistine only tended to attenuate the rotational behavior in the UL rats at doses within the range of clinical use, in agreement with the moderate clinical efficacy in the treatment of vertigo (3). However, we demonstrated that a more specific H_3 antagonist, thioperamide (19), decreased EBRT scores in UL rats more effectively than betahistine. We also showed that GT-2016, another selective H_3 antagonist, had a similar but weaker effect than thioperamide in the UL model. The greater effectiveness of thioperamide may be related to its greater binding affinity to H_3 receptors or its more facile CNS penetration in comparison to GT-2016 (20, 21). The above observations indicate that centrally-acting compounds are also active in this UL model.

As H_3 receptors occur not only as autoreceptors but also as heteroreceptors (22), the effects of H_3 agents might also act through other neurotransmitter systems. H_3 heteroreceptors have been shown to modify the release of acetylcholine (23, 24), noradrenaline (22, 25, 26), serotonin (27), and dopamine (22, 28); the effects of the H_3 antagonists in this study may involve or be modulated by release of these neurotransmitters.

In conclusion, we have demonstrated that the clinically used histaminergic agents astemizole, betahistine, as well as the more recently developed compounds, thioperamide and GT-2016, attenuate the asymmetrical turning behavior induced by unilateral inner ear injection of ethanol in rats, suggesting the use of this model to evaluate drugs for potential antivertigo effects.

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Pharmacological Evaluation of an *In Vivo* Model of Vestibular Dysfunction in the Rat

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SUMMARY

A unilateral microinjection of either histamine or kainic acid was made into the medial vestibular nucleus of rats, eliciting robust barrel rotations that were evaluated by an elevated body-rotation test. Systemic pretreatment with betahistine or GT-2016 significantly attenuated the kainic acid-induced barrel rotations. These data indicate that the animal model described herein may represent a new model to identify novel drugs with potential antivertigo properties. ©1999 Prous Science. All rights reserved.

Key words: Betahistine - GT-2016 - Histamine - Kainic acid - Vertigo

INTRODUCTION

Balance disorders can result from a dysfunction or lesion in either the peripheral or central vestibular system. Secondary to a vestibular lesion is often a sensation of dizziness which is thought to reflect a functional imbalance within the vestibular nuclei (1). Vertigo, a more severe form of dizziness, is commonly associated with vestibular dysfunction. Current pharmacological therapies for vertigo remain unsatisfactory; anticholinergics, antihistamines, benzodiazepines, dopamine (D_2) antagonists, and tricyclic antidepressants are all utilized, but the mechanism of action of these agents in vertigo is poorly understood (1-4).

At the experimental level, there is a lack of *in vivo* models to evaluate novel compounds for the potential treatment of vertigo (5). A majority of the animal studies on vestibular dysfunction have used unilaterally labyrinthectomized animals, in which a disruption of the peripheral vestibular system is achieved by the deafferentation of one labyrinth of the inner ear (6). The dysfunctional vestibular syndrome that is induced by this procedure is behaviorally characterized by asymmetrical ocular-motor and postural symptoms that are recovered in 2-3 days (7, 8). Although this model provides relevant behaviors that lends itself to the study of drug effects, the labyrinthectomy procedure is delicate, time-consuming and can be difficult to reproduce in a reliable fashion.

The present paper describes an *in vivo* model of central vestibular dysfunction to evaluate novel compounds for antivertigo properties. As both the histaminergic and glutaminergic systems have been implicated in central vestibular functioning, histamine and kainic acid were utilized for unilateral microinjections into the medial vestibular nucleus (MVN) of rats (5, 9, 10). The unilateral activation of the central vestibular system by either agent elicited robust barrel rotations and were quantitatively analyzed, demonstrating the involvement of both the histaminergic and glutaminergic systems. As a means of pharmacologically validating this procedure, we tested the ability of the clinically used antivertigo agent betahistine to attenuate kainic acid-induced barrel rotations (11). Based on the reported potential of H_3 antagonists for the treatment of vestibular dysfunction (4), the effect of systemic pretreatment with the selective H_3 antagonist GT-2016 on kainic-induced barrel rotations was also evaluated (12).

MATERIALS AND METHODS

Animals

Male Long-Evans rats weighing 200-225 g upon arrival were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). Rats were individually housed in a climate-controlled room on a 12 h light/dark schedule (lights on at 07:00) with *ad libitum* access to food and water.

Surgery

Rats were anesthetized by i.p. administration of 60 mg/kg pentobarbital (Nembutal, Abbott Laboratories) in a volume of 1.0 ml/kg. The rat was secured in a Kopf stereotaxic apparatus with the incisor bar set at 3.5 mm

below horizontal zero and using blunted ear bars to avoid damage to peripheral vestibular structures. A chronic 22 gauge guide cannula was stereotaxically implanted unilaterally at the following coordinates from bregma: AP -10.6 mm and ML ± 1.6 mm (13). The guide cannula was cut to extend 5.5 mm beyond the base of the cannula's pedestal and was lowered perpendicular to the horizontal plane until the base touched the skull surface such that the tip of the guide cannula rested 3.0 mm above the dorsal aspect of the vestibular nucleus complex, thereby avoiding direct contact and local stimulation prior to microinjection of pharmacological agents. The guide cannula and mounting screws were secured to the skull with cranioplastic cement. A stylet was inserted into the guide cannula to occlude it prior to microinjection. All cannula supplies were obtained from Plastics One (Roanoke, VA, USA). Rats were allowed to recover from surgery for at least 72 h prior to testing.

Microinjection

A 28 gauge injection cannula was bevel cut such that the tip extended 3.0 mm beyond the tip of the guide cannula to reach the MVN at a dorso-ventral coordinate of –8.5 mm from the skull surface. The injection cannula was connected to a Hamilton syringe by PE-50 tubing that delivered a 1.0 µl volume over a 60 s period by a Sage Instruments Pump. The injection cannula was left in place for an additional minute following microinjection to allow diffusion of drug solution away from the tip. Naive rats were used for each experiment and were microinjected only once.

Drugs

Betahistine, histamine and kainic acid (Sigma Chemical Company, St. Louis, MO, USA) were dissolved in saline. GT-2016 (synthesized at Abbott Laboratories) was suspended in a 1% Tween solution. For i.p. pretreatment, drugs were administered 30 min before the microinjection in a volume of 1.0 ml/kg. For microinjections into the MVN, drug solutions were prepared to deliver the appropriate concentration in a volume of 1.0 µl.

Procedure

An elevated body-rotation test (EBRT) was developed to measure the severity of pharmacologically-induced barrel rotations. The EBRT is a modified version of the elevated body-swing test used to evaluate asymmetrical swinging behavior following drug injection into the brain (14). The rat is suspended by the end of its tail 2-3 cm above the tabletop for 30 s. The severity of the barrel rotations are rated based on the latency of the rat to begin rotating: rotation starting in the first 10 s (score 3), in the second 10 s (score 2), in the last 10 s (score 1), while a score of zero is assigned to rats that do not show rotational behavior in the 30 s evaluation. Prior to the microinjection, each rat was evaluated by the EBRT, and any rat that did not have a score of zero was excluded from the study. One min following the microinjection into the MVN, each rat was evaluated and assigned an EBRT score. Thereafter, the rats were evaluated every 10 min for 1 h postmicroinjection.

Histology

A separate set of rats was surgically prepared for a dose-response and time-course study on the histological changes following microinjection of kainic acid in the MVN. One set of rats was sacrificed at 4 h postmicroinjection, and a second set of rats was sacrificed at 24 h postmicroinjection, at which time the rats were deeply anesthetized with 100 mg/kg i.p. pentobarbital prior to being perfused transcardially. The brains were perfusion fixed with physiological saline solution followed by 10% phosphate-buffered formalin. After a 24 h period of immersion fixation in 10% phosphate-buffered formalin, the brains were placed in a tissue slicer with 3 mm gradations. After wet-cutting, the brain slices were processed and embedded in paraffin. Sections from the vestibular nuclear region (6 µg) were cut, mounted and stained with hematoxylin and eosin.

Data evaluation

A 1-h average EBRT score was calculated for each rat. For group comparison, the average EBRT score was analyzed by one-way analysis of variance, followed by the Fisher protected least significant difference test for individual mean comparisons.

RESULTS

The ability of a unilateral microinjection of histamine into the MVN to induce barrel rotations, as measured by the EBRT, can be seen in Figure 1 (upper graph). Both 0.6 and 2.0 µM histamine induced barrel rotations with EBRT scores that were significant from vehicle-injected controls ($F[3,13] = 6.9, p < 0.01$). Robust barrel rotations were observed for at least 4 h after microinjection of 2.0 µM histamine (data not shown). No barrel rotations were observed following microinjection of vehicle or 0.2 µM histamine. Other behavioral changes were observed in the histamine-treated rats, including nystagmus and tilting of the head and body, but these were not quantitatively recorded.

Similar to the previously observed effect of histamine, Figure 1 (lower graph) shows the ability of a unilateral microinjection of kainic acid (0.03-1.0 nM) into the MVN to induce barrel rotations with significantly greater EBRT scores in comparison to the vehicle-injected controls ($F[3,14] = 10.2, p < 0.001$). Microinjection of 0.03 nM kainic acid had a brief and mild effect, as only 2 rats exhibited barrel rotations at 1 min postkainic acid. In contrast, barrel rotations with significant EBRT scores were seen with 0.1 nM kainic acid. Microinjection of

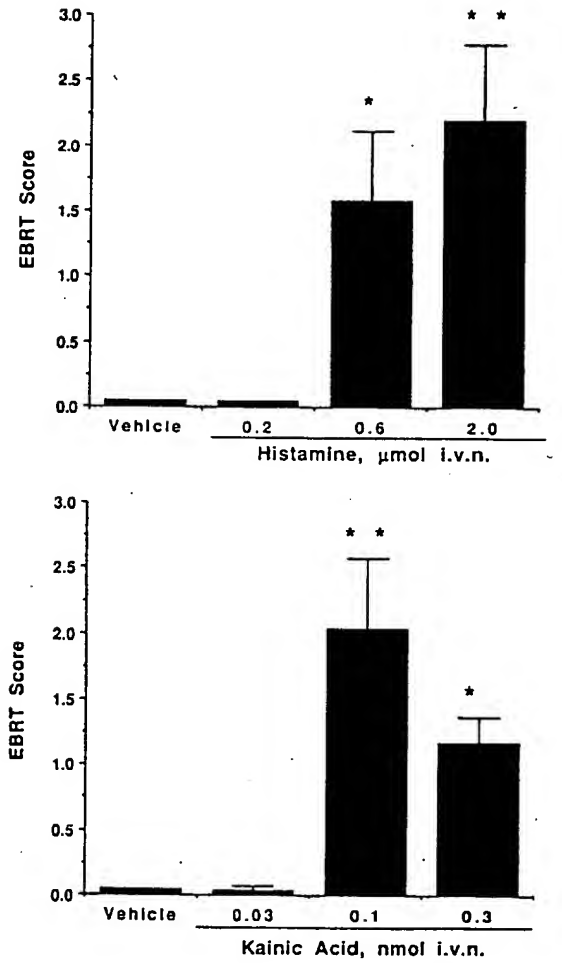

FIG. 1. Unilateral microinjections of histamine into the MVN produces a dose-dependent increase of the elevated body-rotation test (EBRT) score during 1 h after histamine injection. Data represent the mean ± SEM. EBRT score of 4-5 rats per group. $*p < 0.05$; $**p < 0.01$ as compared to vehicle-injected controls (upper graph). Unilateral microinjection of kainic acid into the MVN significantly increased the EBRT score as compared to vehicle-injected controls. Data represent the mean ± SEM. EBRT score of 4-5 rats per group. $*p < 0.05$; $**p < 0.01$ as compared to vehicle-injected controls (lower graph).

0.3 nM kainic acid also induced barrel rotations with significant EBRT scores, however, its effect was less robust than the 0.1 nM group. The reduced ability of 0.3 nM to induce more severe barrel rotations was due to an immobilizing effect on some of the rats in this group. These rats remained immobile when suspended by the tail for the full 30 s evaluation period. At 1.0 nM, all of the rats remained immobile when suspended by the tail (data not shown). Nystagmus and tilting of the head and body were also observed in the kainic acid-treated rats, as was seen in the histamine-treated rats.

Histological examination of the vestibular nuclear region at 4 h necropsy in a separate set of rats microinjected with vehicle or kainic acid (0.03, 0.1 and 0.3 nM) revealed no differences among the groups. Vestibular changes included minimal spongiosis and only occasional axon spheroids. Inflammatory cells were not present within or adjacent to the injection tract and no morphological changes were noted in the vestibular nerve tract, indicating the absence of a lesion in the injected region. In contrast, in rats necropsied at 24 h postinjection of kainic acid (0.03, 0.1 and 0.3 nM), the vestibular nuclear region revealed a mild hemorrhage, substantial spongiosis and moderate axon spheriods. Noteworthy was the finding that 3 of the 4 rats that received the highest dose of kainic acid (0.3 nM) had ischemic neurons in the vestibular nuclear region, indicating the development of a lesion.

Figure 2 (upper graph) shows the significant effect of betahistine pretreatment to attenuate kainic-induced barrel rotations. Kainic acid-treated controls (0.1 nM) exhibited barrel rotations with significantly greater EBRT scores in comparison to vehicle-injected controls ($F[1,18] = 139.4, p < 0.001$), consistent with our findings in the previous figure. Systemic pretreatment with betahistine (14, 48 and 143 µM/kg) before the microinjection of kainic acid significantly attenuated kainic-induced barrel rotations at all doses tested ($F[3,47] = 12.2, p < 0.001$).

Pretreatment with the novel histaminergic ligand GT-2016 also attenuated kainic-induced barrel rotations as shown in Figure 2 (lower graph). Kainic-treated controls exhibited barrel rotations with significantly greater EBRT scores in comparison to vehicle-injected controls ($F[1,18] = 27.7, p < 0.001$). GT-2016 pretreatment (1, 3 and 10 µM/kg i.p.) significantly attenuated the kainic acid-induced barrel rotations at all doses tested ($F[3,47] = 4.3, p < 0.01$).

DISCUSSION

The results of this study demonstrate that histamine and kainic acid induced significant barrel rotations following a unilateral microinjection into the MVN of the rat as measured by the EBRT. The kainic acid-induced barrel rotations were significantly attenuated by systemic pretreatment with the histaminergic ligands betahistine and GT-2016. These data demonstrate that the present animal model of vestibular dysfunction (intravestibular injection of kainic acid) in combination with the EBRT, could be useful to identify novel agents for the treatment of vertigo.

The discovery of new drugs for the treatment of vertigo has been hampered by the lack of animal models that can be easily reproduced. Current models to study vestibular dysfunction rely on electrophysiological methods that are time-consuming, or delicate surgical procedures, such as the unilateral labyrinthectomy. An animal model based on unilateral intravestibular injection of pharmacological agents that disrupt central vestibular functioning is hereby described.

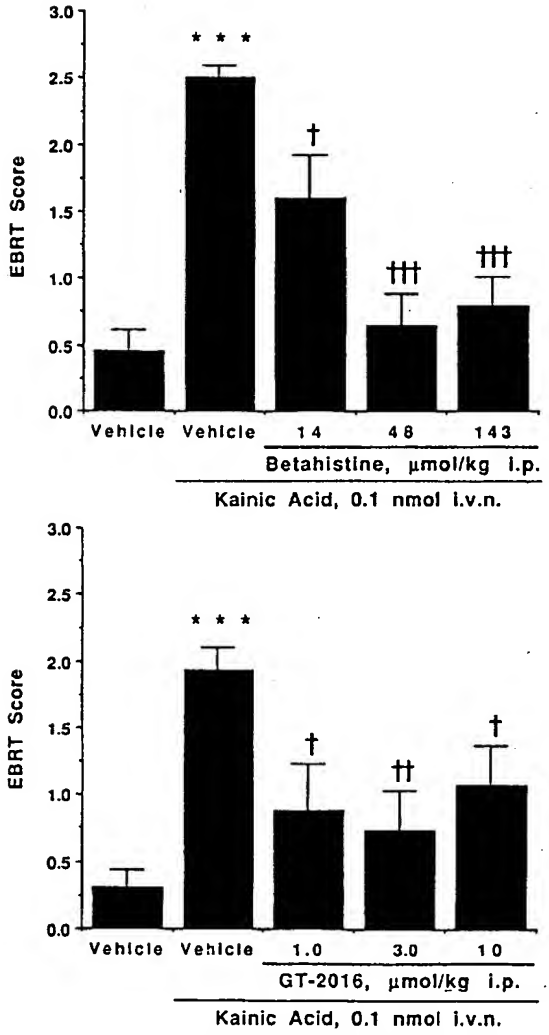

FIG. 2. Systemic pretreatment with betahistine significantly attenuated the increased EBRT score induced by unilateral microinjection of 0.1 nM kainic acid into the MVN. Data represent the mean ± SEM. EBRT score of 8-13 rats per group. Rats were injected i.p. with vehicle or betahistine (14, 48 or 143 µM/kg) 30 min before microinjection of vehicle or kainic acid into the MVN. ***$p < 0.001$ as compared to vehicle-injected controls. †$p < 0.05$; †††$p < 0.001$ as compared to kainic acid-treated controls (upper graph). Systemic pretreatment with GT-2016 significantly attenuated the increased EBRT score induced by unilateral microinjection of 0.1 nM kainic acid into the MVN. Data represent the mean ± SEM. EBRT score of 8-15 rats per group. Rats were injected i.p. with vehicle or GT-2016 (1, 3 or 10 µM/kg) 30 min before microinjection of vehicle or kainic acid into the MVN. ***$p < 0.001$ as compared to vehicle-injected controls. †$p < 0.05$; ††$p < 0.01$ as compared to kainic acid-treated controls (lower graph).

The present investigation demonstrated that both histamine and kainic acid induced significant barrel rotations, a behavioral change that is also observed following a unilateral labyrinthectomy. Smith (15) has shown that following unilateral labyrinthectomy in guinea pigs, there is a dramatic loss of resting activity in the vestibular neurons ipsilateral to the lesion, most notably in the MVN. This ipsilateral decrease in activity creates in an imbalance with the contralateral vestibular nuclei and is accompanied by asymmetrical behavioral symptoms. Some of the asymmetrical symptoms that have been described include spontaneous nystagmus, tilting of the head or the whole body and barrel rotating toward the deafferented side in the roll plane (7, 8, 16). The similarities between the asymmetrical behavioral syndromes induced by either unilateral labyrinthectomy or intravestibular microinjection indicates that the pharmacologically-induced impairments we observed could similarly be the result of an imbalance of neuronal activity between the central vestibular nuclei due to their excitatory effect on MVN neurons (1, 17).

Based on substantial evidence for the presence of pre- and postsynaptic histaminergic receptors in the MVN (9, 10) we initially evaluated the effect of a unilateral microinjection of histamine in the MVN. Histamine induced spontaneous nystagmus, head and body tilt and robust barrel rotations about the long axis of the body, consistent with the behavioral changes following unilateral labyrinthectomy.

The glutaminergic system has been implicated in vestibular system functioning (5) and it has been demonstrated that kainic acid microinjected into the vestibular nucleus activates the central vestibular system (18). The present study demonstrates that unilateral microinjection of kainic acid induced barrel rotations which resulted in significant EBRT scores. The known excitatory effect of kainic acid in the vestibular nuclei and the induced barrel rotations lend support to the hypothesis of a pharmacologically induced imbalance of neuronal vestibular activity underlying the behavioral vestibular impairment.

In a separate study, histological examination of the vestibular nuclear region at 4 h postmicroinjection of kainic acid, a time point at which most kainic-treated rats have ceased barrel rotating, revealed no demonstrable differences between the vehicle-injected controls and kainic acid-treated rats. It is therefore unlikely that the observed kainic-induced behavioral impairment was due to cell death of MVN neurons as there were no evident morphological changes in the vestibular region until 24 h postmicroinjection, but rather that the imbalance was due to a unilateral excitatory effect of the kainic acid.

In an effort to assess the validity of the intravestibular kainic acid model for evaluating novel antivertigo compounds, the ability of betahistine to attenuate the kainic acid-induced barrel rotations was investigated. Systemic pretreatment with betahistine significantly attenuated the kainic-induced barrel rotations at 14, 48 and 143 µM/kg, giving pharmacological validation to this model.

The demonstration that betahistine pretreatment attenuated the kainic acid-induced barrel rotations

suggests that it is able to reduce the imbalanced vestibular activity induced by unilateral excitation of kainic acid. Betahistine has been shown to reduce the excitability and sensitivity of vestibular neurons to the excitatory effect of histamine and may have a similar effect against the excitatory effect of kainic acid (19). The mechanism by which betahistine exerts its effect against kainic acid is not clear from the present study.

As the mechanism by which betahistine produces its beneficial clinical action has been attributed to its antagonism at the H_3 autoreceptor (4), we evaluated the ability of the more selective H_3 antagonist GT-2016 to attenuate the barrel rotations induced by unilateral microinjection of kainic acid in the MVN. Systemic pretreatment with GT-2016 had an attenuating effect against the kainic acid-induced barrel rotations, supporting the notion that betahistine exerts some of its effect through H_3 receptor blockade.

In conclusion, our finding that betahistine, which has been used in the clinical treatment of vestibular disorders, was effective in attenuating the barrel rotations induced by unilateral microinjection of kainic acid into the MVN of rats as measured by the EBRT, lends support to the use of this model for evaluating novel compounds for potential treatment of vertigo. Further, the efficacy of GT-2016 in this model provides support of the potential of selective H_3 antagonists in the treatment of vestibular disorders.

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Role of histamine in rodent antinociception

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1 Effects of substances which are able to alter brain histamine levels on the nociceptive threshold were investigated in mice and rats by means of tests inducing three different kinds of noxious stimuli: mechanical (paw pressure), chemical (abdominal constriction) and thermal (hot plate).

2 A wide range of i.c.v. doses of histamine 2HCl was studied. Relatively high doses were dose-dependently antinociceptive in all three tests: 5–100 μg per rat in the paw pressure test, 5–50 μg per mouse in the abdominal constriction test and 50–100 μg per mouse in the hot plate test. Conversely, very low doses were hyperalgesic: 0.5 μg per rat in the paw pressure test and 0.1–1 μg per mouse in the hot plate test. In the abdominal constriction test no hyperalgesic effect was observed.

3 The histamine H_3 antagonist, thioperamide maleate, elicited a weak but statistically significant dose-dependent antinociceptive effect by both parenteral (10–40 mg kg^{-1}) and i.c.v. (1.1–10 μg per rat and 3.4–10 μg per mouse) routes.

4 The histamine H_3 agonist, (R)-α-methylhistamine dihydrogenomaleate was hyperalgesic, with a rapid effect (15 min after treatment) following i.c.v. administration of 1 μg per rat and 3 μg per mouse, or i.p. administration of 100 mg kg^{-1} in mice. In rats 20 mg kg^{-1}, i.p., elicited hyperalgesia only 4 h after treatment.

5 Thioperamide-induced antinociception was completely prevented by pretreatment with a non-hyperalgesic i.p. dose of (R)-α-methylhistamine in the mouse hot plate and abdominal constriction tests. Antagonism was also observed when both substances were administered i.c.v. in rats.

6 L-Histidine HCl dose-dependently induced a slowly occurring antinociception in all three tests. The doses of 250 and 500 mg kg^{-1}, i.p. were effective in the rat paw pressure test, and those of 500 and 1500 mg kg^{-1}, i.p. in the mouse hot plate test. In the mouse abdominal constriction test 500 and 1000 mg kg^{-1}, i.p. showed their maximum effect 2 h after treatment.

7 The histamine N-methyltransferase inhibitor, metoprine, elicited a long-lasting, dose-dependent antinociception in all three tests by both i.p. (10–30 mg kg^{-1}) and i.c.v. (50–100 μg per rat) routes.

8 To ascertain the mechanism of action of the antinociceptive effect of L-histidine and metoprine, the two substances were also studied in combination with the histamine synthesis inhibitor (S)-α-fluoromethylhistidine and with (R)-α-methylhistamine, respectively. L-Histidine antinociception was completely antagonized in all three tests by pretreatment with (S)-α-fluoromethylhistidine HCl (50 mg kg^{-1}, i.p.) administered 2 h before L-histidine treatment. Similarly, metoprine antinociception was prevented by (R)-α-methylhistamine dihydrogenomaleate 20 mg kg^{-1}, i.p. administered 15 min before metoprine. Both (S)-α-fluoromethylhistidine and (R)-α-methylhistamine were used at doses which did not modify the nociceptive threshold when given alone.

9 The catabolism product, 1-methylhistamine, administered i.c.v. had no effect in either rat paw pressure or mouse abdominal constriction tests.

10 These results indicate that the antinociceptive action of histamine may take place on the postsynaptic site, and that its hyperalgesic effect occurs with low doses acting on the presynaptic receptor. This hypothesis is supported by the fact that the H_3 antagonist, thioperamide is antinociceptive and the H_3 agonist, (R)-α-methylhistamine is hyperalgesic, probably modulating endogenous histamine release. L-Histidine and metoprine, which are both able to increase brain histamine levels, are also able to induce antinociception in mice and rats. Involvement of the histaminergic system in the modulation of nociceptive stimuli is thus proposed.

Keywords: Analgesia; antinociception; pain; histamine; L-histidine; (R)-α-methylhistamine; thioperamide; metoprine; (S)-α-fluoromethylhistidine; methylhistamine

Introduction

Modulation of nociception can occur via different neuronal systems. Many neuromediators besides enkephalins, such as acetylcholine (ACh) (Metys *et al.*, 1969; Bartolini *et al.*, 1992), γ-aminobutyric acid (GABA) (Liebman & Pastor, 1980; Malcangio *et al.*, 1992), catecholamines (Jones & Gebhart, 1986) and 5-hydroxytryptamine (5-HT) (Samanin & Valzelli, 1971) have been reported to be involved in nociception control. Recently histamine, which is regarded as an autacoid associated with cutaneous pain (Crossland, 1980), has also been shown to take part in antinociception. Intracerebroventricular (i.c.v.) administration elicits antinociception in both rats (Glick & Crane, 1978; Bhattacharya & Parmar, 1985; Parolaro *et al.*, 1989) and mice (Chung *et al.*, 1984; Oluyomi & Hart, 1991) at relatively high doses. Glick & Crane (1978) also reported that injection of histamine into the rat dorsal raphe nucleus and periaqueductal grey region caused antinociception, while its injection into the median raphe nucleus caused hyperalgesia.

Conversely, the role of endogenous histamine in antinociception has not yet been investigated. Its importance has grown in the last few years with the discovery and definition of a histaminergic neuronal system in mammalian brain. According to many research groups (Steinbusch & Mulder,

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1985; Panula *et al.*, 1989; Wada *et al.*, 1991), in addition to two ascending histaminergic pathways, there is a minor descending pathway which arises from hypothalamic neurones. Its fibres can be found in the dorsal raphe nucleus and periaqueductal grey region, areas which are considered to be important for pain modulation (Basbaum & Fields, 1984).

Furthermore, the existence of presynaptic histamine receptors, called H_3, was reported by Arrang *et al.* (1983). According to the authors, their stimulation inhibits histamine release (Arrang *et al.*, 1983; Van der Werf *et al.*, 1987) and synthesis (Arrang *et al.*, 1987b). Lately, this same group described the effects of a potent and selective H_3 receptor agonist, (R)-α-methylhistamine (RAMH), and an antagonist, thioperamide (Arrang *et al.*, 1987a; Garbarg *et al.*, 1989). The two molecules were seen to be good tools for studying the role of endogenous histamine.

Besides investigating the effects of a wide range of histamine doses, or acting on the histamine H_3 receptor with RAMH and thioperamide, a further way to study the role of endogenous histamine in antinociception might be to alter histamine brain levels. Different substances such as the histamine precursor, L-histidine, the histidine decarboxylase [EC 4.1.1.22] (HDC), (S)-α-fluoromethylhistidine (FMH) (Kollonitsch *et al.*, 1978) and the histamine-*N*-methyltransferase [EC 2.1.1.8] (HMT) inhibitor, metoprine (Duch *et al.*, 1978), have in fact been described as able to alter selectively histamine brain levels. Oluyomi & Hart (1991) recently reported an antinociceptive effect for histidine, as well as for thioperamide, in the mouse hot plate test, while there are no reports on the effects of histamine synthesis or catabolism inhibitors.

We therefore considered it worthwhile investigating the role of the histaminergic system in antinociception by using all three of the aforementioned strategies in both mice and rats, with three different kinds of antinociceptive tests.

Preliminary data were presented at the XXIth and XXIIth Annual Meetings of the European Histamine Research Society, (Lamberti *et al.*, 1992b; Malmberg-Aiello *et al.*, 1992; 1993) and at the IXth Meeting of the European Society for Neurochemistry (Lamberti *et al.*, 1992a).

Methods

Male Swiss-Webster mice (22–28 g) and Wistar rats (120–180 g) were used. Fifteen mice or four rats were housed per cage. The cages were taken to the experimental room 24 h before the experiment, for acclimatization. The animals were fed *ad libitum* a standard laboratory diet and tap water.

Hot plate test

The method described by O'Callaghan & Holtzman (1976) was adopted, using a stainless steel container (36 × 28 × 30 cm), thermostatically set at 52.5 ± 0.1°C, in a precision water-bath. Mice with a licking latency below 12 and over 18 s in the test before drug administration (30%) were rejected. An arbitrary cut-off time of 45 s was adopted.

Abdominal constriction test

The test was performed in mice according to Koster *et al.* (1959). The number of stretching movements was counted for 10 min, starting 5 min after 0.6% acetic acid injection.

Paw pressure test

The nociceptive threshold in rats was determined with an analgesymeter (Ugo Basile, Varese, Italy) according to the method described by Leighton *et al.* (1988). Rats scoring below 50 g or over 80 g during the test before drug administration (25%) were rejected. An arbitrary cut-off value of 250 g was adopted.

Rota-rod test

The integrity of motor coordination was assessed on the basis of the endurance time of the animals on the rotating rod according to Kuribara *et al.* (1977). On the day of the test, the performance time was measured before and 15 min after treatment.

Drugs

The following drugs were used: histamine dihydrochloride, L-histidine monohydrochloride monohydrate and 1-methylhistamine dihydrochloride (Sigma); (R)-α-methylhistamine dihydrogenomaleate (Bioprojet), (S)-α-fluoromethylhistidine monohydrochloride (Merck Sharp & Dohme Research Lab.), morphine hydrochloride (USL 10/D), metoprine (Burroughs Wellcome) and thioperamide maleate (RBI). The doses given in the text are expressed as salts. All drugs except metoprine were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Metoprine was dissolved in 10% aqueous lactic acid and then diluted with saline (1:30). Drug concentrations were prepared in such a way that the necessary dose could be injected in a volume of 10 ml kg^{-1} by both s.c. and i.p. route.

I.c.v. administration was performed in two different ways. For the first method a short ether anaesthesia was adopted. Substances were injected in the necessary dose dissolved in 5 μl for mice and in 10 μl for rats, according to the method described by Haley & McCormick (1957) for mice and extended to rats by us. The second approach consisted of injecting the substances in conscious rats with permanent i.c.v. polyethylene cannulae (5 μl of drug solution + 2 μl air + 5 μl saline) implanted according to the method described by Altaffer *et al.* (1970), in order to avoid false responses due to the effect of ether. To ascertain the exact site of i.c.v. injection, some mice or rats were injected i.c.v. with 5 μl or 10 μl of 1:10 diluted Indian ink and their brains were examined macroscopically after sectioning.

Statistical analysis

Results are given as the mean ± s.e. Student's two-tailed *t* test was used to verify significance between two means. *P* values of less than 0.05 were considered significant. Multiple comparisons with appropriate controls were made with ANOVA, followed by the multiple range test for least significant differences (LSD). Means with 95% confidence intervals that did not overlap were considered significantly different. ED_{30} and ED_{30} values are the doses which produced respectively the 50% and 30% of the maximum possible effect with 95% confidence limits. Data were analyzed with computer programmes (Tallarida & Murray, 1984, and STATGRAPHICS, 1986, STSC Inc. U.S.A.).

Results

Histamine antinociceptive and hyperalgesic effects

Histamine was administered i.c.v. in doses ranging from 0.05 to 100 μg per mouse and from 0.1 to 100 μg per rat. Very low doses (0.1, 0.5 and 1 μg per mouse) induced significant hyperalgesia 15 min after treatment, while high doses (50 and 100 μg per mouse) elicited antinociception which was still evident 45 min after treatment in the hot plate test (Figure 1a). The ED_{30} for the antinociceptive effect (15 min after treatment) was 96.0 (71.9–144.7) μg per mouse i.c.v.

The same biphasic effect was observed in the paw pressure test on rats. Histamine caused statistically-significant hyperalgesia 30 min after treatment at 0.5 μg per rat administered i.c.v. during ether anaesthesia and at both 0.5 and 1 μg per rat i.c.v., 45 min after treatment; antinociception was detectable at doses of 5–50 μg per rat (Figure 2a). In order to

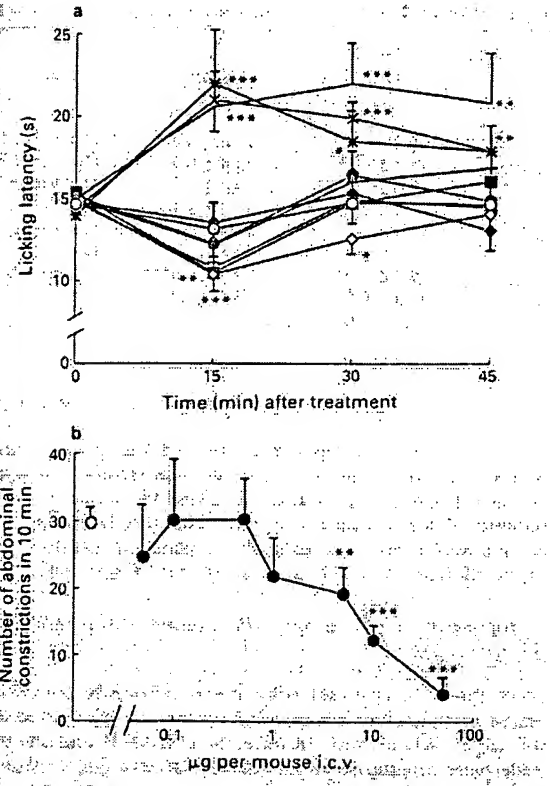

Figure 1 Effect of i.c.v. histamine 2HCl on the nociceptive threshold in mice. (a) Biphasic effect in the hot plate test. Groups were treated (μg per mouse i.c.v.) as follows: saline 5 μl (○); histamine 0.05 (●); 0.1 (□); 0.5 (■); 1 (◇); 5 (◆); 10 (+); 50 (×) and 100 (*). In comparison, the effect of morphine HCl 5 mg kg^{-1}, s.c. (no symbol) is shown. (b) Antinociceptive effect in the abdominal constriction test. Saline (○) and histamine (●) were injected 15 min before the test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus saline controls. Each point represents the mean (with s.e.mean) of 11–79 mice.

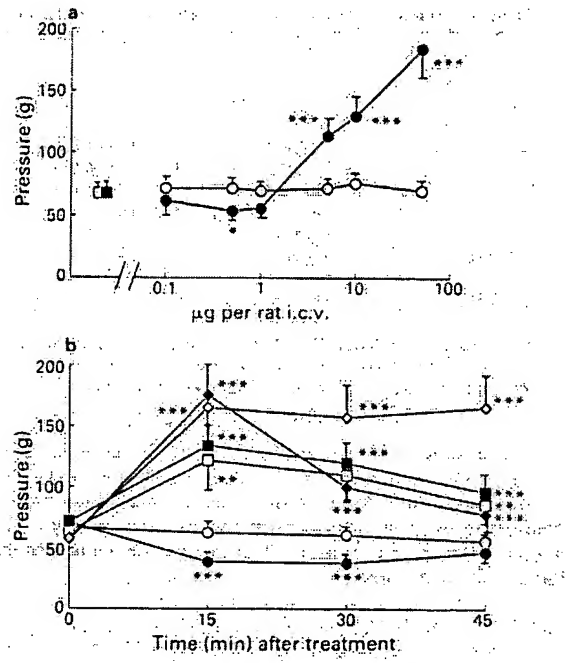

Figure 2 Biphasic effect of histamine on the nociceptive threshold in the rat paw pressure test. (a) Dose-response curve of histamine effect. Saline 10 μl per rat (○) and histamine 2HCl (●) were administered by i.c.v. injection during short ether anaesthesia, 30 min before test. Symbols (□) and (■) indicate pretest values of saline and histamine-treated groups respectively. (b) Effects on rats with permanent i.c.v. cannulae. Groups were (dose per rat i.c.v.): saline 5 μl (○); histamine 2HCl 0.5 μg (●), 10 μg (□), 50 μg (■), 100 μg (◆). In comparison the effect of morphine 5 mg kg^{-1}, s.c. (◇) is shown. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus saline controls. Each point represents the mean (with s.e.mean) of 7–23 rats.

make sure that ether anaesthesia did not affect the results hyperalgesic and antinociceptive doses of histamine were also administered to conscious rats via permanent i.c.v. cannulae. The results obtained confirmed our previous observations and revealed a maximum effect at 15 min (Figure 2b), with an ED_{50} of 69.0 (13.3–356.9) μg per rat i.c.v.

In the mouse abdominal constriction test only the antinociceptive effect was detectable. Histamine induced a statistically-significant antinociception at 5, 10 and 50 μg per mouse with an ED_{50} of 6.7 (3.8–11.7) μg per mouse i.c.v. (Figure 1b). The antinociception induced by 50 μg per mouse is comparable to that induced by 2 mg kg^{-1} of morphine HCl (5.4 ± 2.0 constrictions 15 min after s.c. administration), and a comparison with the effects of morphine in the hot plate and paw pressure tests is given in Figures 1a and 2b, respectively.

The histamine dose of 50 μg per mouse was also used to study mouse rota-rod performance 15 min after treatment. No effect was seen on endurance time on the rod in histamine- and saline-treated mice (186 ± 40 s versus 229 ± 23 s of controls).

Thioperamide antinociceptive effect

In all three tests thioperamide was able to induce a statistically-significant antinociception. Both i.c.v. and parenteral routes of administration were used.

In the mouse hot plate test, 20 mg kg^{-1}, i.p. appeared to be the optimum dose for eliciting antinociception (Table 1), which persisted up to 30 min with a maximum response at 15 min. A lower (10 mg kg^{-1}, i.p.) or higher (40 mg kg^{-1}, i.p.) dose was less effective in raising the nociceptive threshold. When given i.c.v. in doses ranging from 0.5 to 10 μg per mouse, no statistically-significant effect was observed for thioperamide (data not shown).

Conversely, in the mouse abdominal constriction test, i.c.v. administration of thioperamide showed an initial dose-dependent action with a maximum effect at 10 μg per mouse, while a higher dose (30 μg per mouse) was not effective in significantly decreasing the number of abdominal constrictions (Figure 3a). Similarly, subcutaneous administration revealed a statistically-significant antinociception only for the same dose which elicited the maximum effect in the hot plate test (Figure 3b).

Thioperamide antinociception was confirmed in the rat paw pressure test, where the time-course for the dose of 20 mg kg^{-1}, i.p. reflected the one observed with the hot plate test (Figure 4a). Both 1.1 and 10 μg per rat administered via permanent i.c.v. cannulae were antinociceptive; the effect of the latter was still significant 2 h after treatment (Table 2).

Effect of (R)-α-methylhistamine (RAMH) on nociceptive threshold

When given at sufficiently high doses, RAMH was able to induce hyperalgesia in the hot plate and paw pressure tests.

In the mouse hot plate test, three different i.p. doses were used. The doses of 5 and 20 mg kg^{-1} were ineffective in modifying the nociceptive threshold, while 100 mg kg^{-1} elicited a highly significant hyperalgesia up to 60 min after treatment (Table 1). The dose 20 mg kg^{-1}, i.p. also had no effect in the abdominal constriction test (Figure 3b).

Table 1 Effects of thioperamide and (R)-α-methylhistamine (RAMH) i.p. alone and combined in the mouse hot plate test

Pretreatment (mg kg^{-1}, i.p.)	Treatment (mg kg^{-1}, i.p.)	n	Licking latency (s) Pretest	15 min after treatment	30 min after treatment	45 min after treatment	60 min after treatment
Saline 10 ml	Saline 10 ml	47	14.6 ± 0.3	13.5 ± 0.4	12.9 ± 0.4	13.6 ± 0.4	13.9 ± 0.4
	Thioper 10	14	14.4 ± 0.4	16.0 ± 1.4*	15.3 ± 1.0*	14.0 ± 0.9	13.1 ± 0.7
Saline	Thioper 20	12	14.6 ± 0.3	19.6 ± 0.9***	18.3 ± 1.5***	15.5 ± 1.5	14.3 ± 0.7
	Thioper 40	14	14.5 ± 0.3	16.0 ± 1.0**	15.0 ± 1.1*	13.7 ± 1.1	14.2 ± 0.8
	RAMH 5	5	14.6 ± 1.1	12.4 ± 1.4	12.4 ± 1.1	12.2 ± 0.9	14.8 ± 0.7
RAMH 20	Saline	30	14.3 ± 0.4	12.2 ± 0.9	13.1 ± 0.7	13.5 ± 0.6	14.6 ± 1.1
	RAMH 100	11	14.5 ± 0.6	9.6 ± 0.8***	9.7 ± 1.0**	9.4 ± 1.0***	11.6 ± 0.9*
RAMH 20	Thioper 20	10	14.5 ± 0.6	12.7 ± 0.8^^	13.4 ± 0.9^	14.4 ± 1.2	13.0 ± 0.8

*$P<0.05$; **$P<0.01$; ***$P<0.001$ versus saline controls.
^$P<0.02$; ^^$P<0.001$ versus thioperamide (20 mg kg^{-1})-treated mice.
Pretreatment was performed 15 min before treatment.

A wide range of doses, from 1 to 100 µg per mouse, was tested by i.c.v. route in the hot plate test, but only 3 µg per mouse significantly lowered the latency from 13.0 ± 0.5 s of controls to 9.7 ± 0.6 s in 17 mice 15 min after treatment. The highest doses tested, 50 and 100 µg per mouse, caused immobility and convulsions.

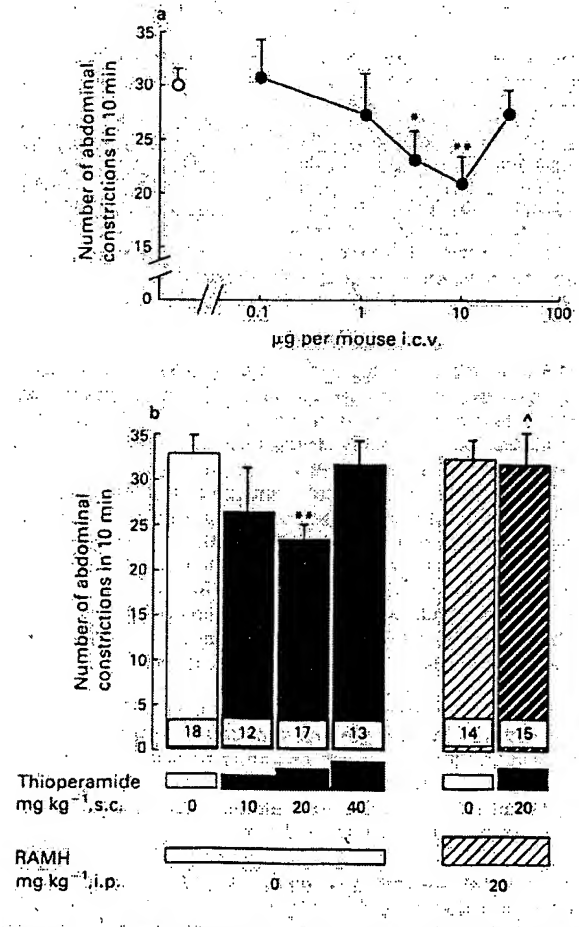

Figure 3 Antinociceptive effect of i.c.v. and s.c. thioperamide in the mouse abdominal constriction test. (a) Thioperamide maleate was administered i.c.v. 15 min before test. Each point represents the mean of 12–19 mice. (b) Antinociceptive effect of thioperamide and its antagonism by (R)-α-methylhistamine (RAMH). RAMH dihydrogenomaleate was administered 15 min before thioperamide and thioperamide s.c. 15 min before test. Inside the columns is shown the number of mice. Vertical lines give s.e.mean. *$P<0.02$; **$P<0.01$ versus saline controls. ^$P<0.05$ versus thioperamide (20 mg kg^{-1})-treated mice.

Similarly, in the rat paw pressure test 1 µg per rat administered i.c.v. via permanent cannulae was significantly hyperalgesic (Table 2). The dose of 20 mg kg^{-1} was tested by parenteral administration in rats. The threshold was gradually lowered, and a statistically-significant hyperalgesia was observed from 4 to 7 h after treatment (Figure 4b).

Antagonism by (R)-α-methylhistamine of thioperamide antinociception

In all three tests RAMH administered 15 min before thioperamide completely prevented the antinociception induced by the latter. When tested in mice, both RAMH and thioperamide were administered parenterally at 20 mg kg^{-1} (Table 1 and Figure 3b), while in the rat paw pressure test i.c.v. administration via permanent cannulae was adopted (Table 2).

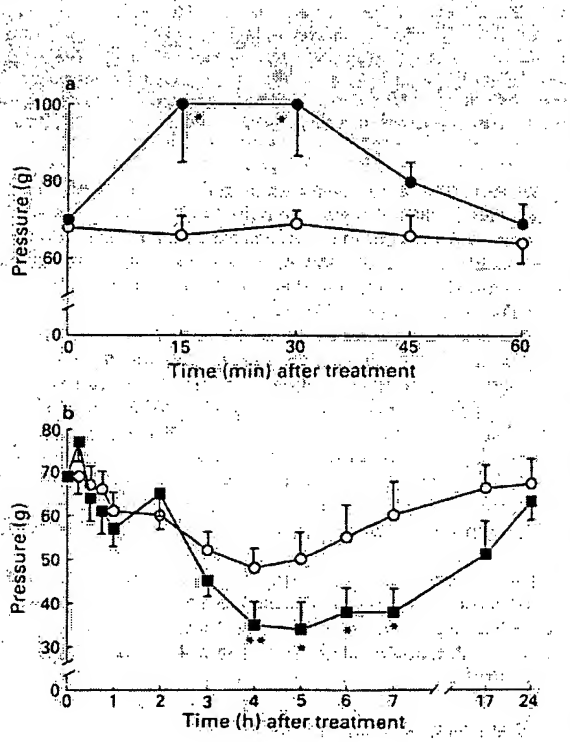

Figure 4 Effects of thioperamide and (R)-α-methylhistamine (RAMH) on nociceptive threshold in the rat paw pressure test. (a) Antinociceptive effect of thioperamide maleate 20 mg kg^{-1}, i.p. (●) and (b) hyperalgesic effect of RAMH dihydrogenomaleate 20 mg kg^{-1}, i.p. (■) in comparison with saline-treated (○) rats. Each point represents the mean with s.e.mean of 7–11 rats. *$P<0.05$; **$P<0.01$ versus saline controls.

Table 2 Effects of thioperamide and (R)-α-methylhistamine (RAMH) i.c.v. alone and combined in the rat paw pressure test

Pretreatment (μg per rat)	Treatment (μg per rat)	n	Pressure (g) Pretest	15 min	30 min	45 min after treatment	60 min	120 min
Saline 10 μl	Saline 10 μl	19	60 ± 2	60 ± 2	58 ± 1	57 ± 2	57 ± 2	57 ± 3
	Thioper. 1.1	9	60 ± 2	87 ± 6***	77 ± 6**	63 ± 4	56 ± 2	–
Saline	Thioper. 10	10	60 ± 2	102 ± 10***	84 ± 6***	68 ± 4*	66 ± 3*	71 ± 3**
RAMH 1	Saline	14	63 ± 1	49 ± 3**	54 ± 2	46 ± 2*	47 ± 2**	45 ± 2**
RAMH 1	Thioper. 10	12	58 ± 2	64 ± 2^^	58 ± 2^^	56 ± 2^	58 ± 2^	–

*$P<0.05$; **$P<0.01$; ***$P<0.001$ versus saline-treated rats.
^$P<0.05$; ^^$P<0.001$ versus thioperamide (10 μg)-treated rats.
Pretreatment was performed 15 min before treatment.

L-Histidine antinociceptive effect

L-Histidine was able to raise significantly the nociceptive threshold in both rats and mice in all three tests.

In the rat paw pressure test the antinociceptive effect was dose-dependent and for both doses used (250 and 500 mg kg^{-1}, i.p.), it lasted until 3 h after treatment, the highest one producing a peak effect 2 h after administration (Figure 5).

In the mouse abdominal constriction test also, histidine antinociception followed a pattern which was both time- and dose-dependent. Both doses used in this test, 500 and 1000 mg kg^{-1} i.p., decreased the number of abdominal constrictions with a maximum effect 2 h after treatment. At this same time the percentage of inhibition was 34% and 69% respectively (Figure 6a).

In the mouse hot plate test the dose-dependent rise in the nociceptive threshold was very small but significant, and for the highest dose (1500 mg kg^{-1}, i.p.) was detectable from 30 to 180 min after treatment (Figure 6b). Despite this high dose, the antinociception obtained produced no visible change in the animals' normal behaviour.

Antagonism of histidine-induced antinociception by (S)-α-fluoromethylhistidine (FMH)

In all three tests FMH, administered 2 h before histidine treatment, was able to prevent completely the antinociception induced by the latter (Figures 5 and 6). FMH was given at a dose (50 mg kg^{-1}, i.p.) which neither modified the nociceptive threshold when given alone, nor altered normal animal behaviour.

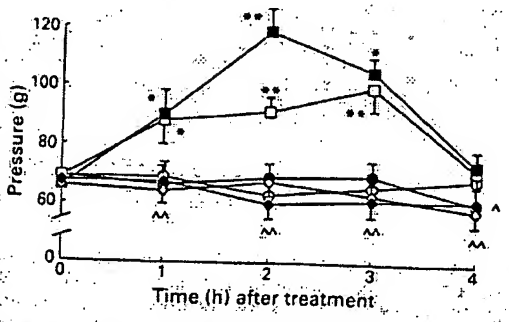

Figure 5 Antinociceptive effect of L-histidine and its antagonism by (S)-α-fluoromethylhistidine (FMH) in the rat paw pressure test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml $10\ g^{-1}$ + saline (○); FMH HCl 50 mg kg^{-1} + saline (●); saline + L-histidine HCl 250 mg kg^{-1} (□); saline + L-histidine 500 mg kg^{-1} (■); FMH 50 mg kg^{-1} + L-histidine 250 mg kg^{-1} (◇); FMH 50 mg kg^{-1} + L-histidine 500 mg kg^{-1} (◆). Pretreatment was performed 2 h before treatment. *$P<0.01$; **$P<0.001$ versus saline controls. ^$P<0.05$; ^^$P<0.01$ versus L-histidine-treated mice. Each point represents the mean (with s.e.mean) of 9–20 rats.

Metoprine antinociceptive effect

In the mouse hot plate test, doses of 5, 20 and 30 mg kg^{-1}, i.p. of metoprine were studied. The antinociception observed was dose-dependent: 5 mg kg^{-1} was ineffective, while at 20 mg kg^{-1} the antinociceptive effect was significant for 1 h and at 30 mg kg^{-1} a very strong effect lasted up to 24 h (Figure 7a). Mice treated with the highest dose showed slight excitation inside their cages; this became more noticeable when they were placed on the plate. Calculations revealed an ED_{50} of 19.5 (13.5–28.3) mg kg^{-1}, i.p. 15 min after treatment.

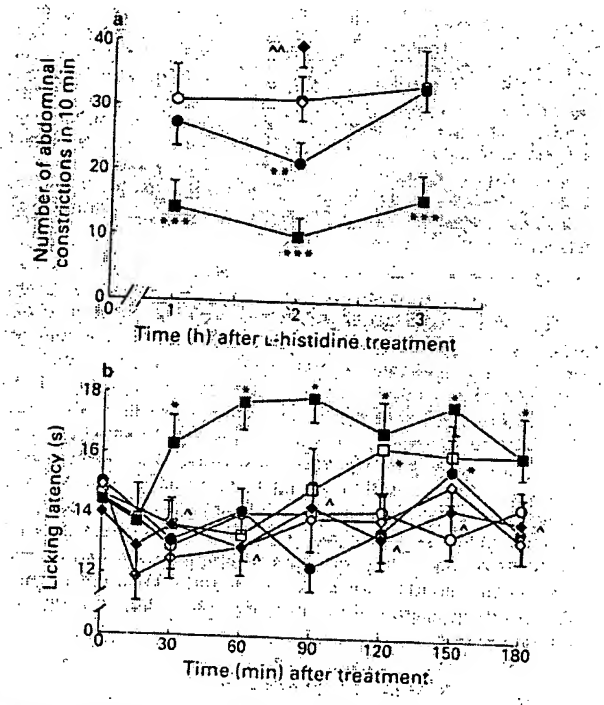

Figure 6 Time course of L-histidine antinociception and its antagonism by (S)-α-fluoromethylhistidine (FMH) in mice. (a) Abdominal constriction test. FMH was administered 2 h before L-histidine and L-histidine 1, 2 or 3 h before test. Groups (pretreatment i.p. + treatment i.p.) were: saline 0.1 ml $10\ g^{-1}$ + saline (○); saline + L-histidine HCl 500 mg kg^{-1} (●); L-histidine 1000 mg kg^{-1} (■); FMH HCl 50 mg kg^{-1} + saline (◇); FMH + L-histidine 500 mg kg^{-1} (◆). Each point represents the mean of 9–16 mice. **$P<0.01$; ***$P<0.001$ versus saline controls. ^$P<0.001$ versus L-histidine 500 mg kg^{-1} (2 h before test)-treated mice. (b) Hot plate test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml $10\ g^{-1}$ + saline (○); L-histidine 250 mg kg^{-1} (●); L-histidine 500 mg kg^{-1} (□); saline + L-histidine 1500 mg kg^{-1} (■); FMH 50 mg kg^{-1} + saline (◇); FMH 50 mg kg^{-1} + L-histidine 1500 mg kg^{-1} (◆). Pretreatment was performed 2 h before treatment. Each point represents the mean of 12–26 mice. *$P<0.05$ versus saline controls. ^$P<0.05$ versus L-histidine (1500 mg kg^{-1})-treated mice.

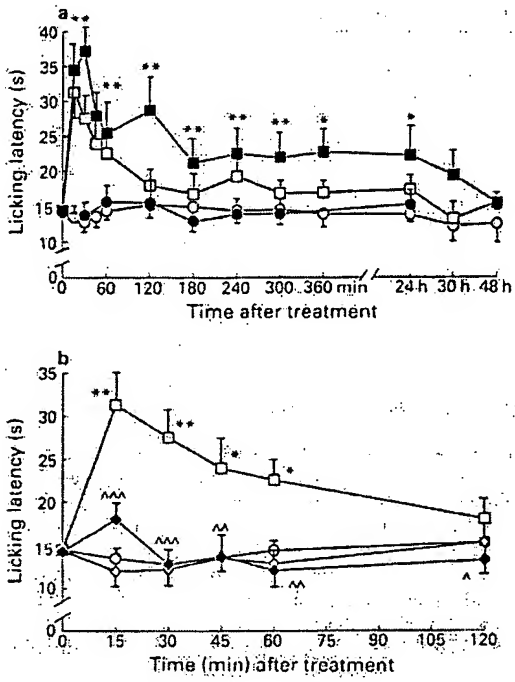

Figure 7 Antinociceptive effect of metoprine (a) and its antagonism by (R)-α-methylhistamine (RAMH) (b) in the mouse hot plate test. Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml $10\,g^{-1}$ + lactic acid 0.3% in saline (○); metoprine 5 mg kg^{-1} (●); saline + metoprine 20 mg kg^{-1} (□); metoprine 30 mg kg^{-1} (■); RAMH dihydrogenomaleate 20 mg kg^{-1} + lactic acid 0.3% (◇); RAMH + metoprine 20 mg kg^{-1} (◆). Pretreatment was performed 15 min before treatment. $^{*}P<0.01$; $^{**}P<0.001$ versus saline controls. $^{\wedge}P<0.05$; $^{\wedge\wedge}P<0.01$; $^{\wedge\wedge\wedge}P<0.001$ versus metoprine (20 mg kg^{-1})-treated mice. Each point represents the mean (with s.e.mean) of 11–24 mice.

The same dose-dependent antinociceptive effect was observed in the mouse abdominal constriction test. The number of stretching movements was significantly reduced with 10 and 20 mg kg^{-1}, i.p. 15–25 min after treatment with an ED_{50} of 15.3 (10.3–22.8) mg kg^{-1}, i.p. and 20 min later the effect for the dose 20 mg kg^{-1} was even more significant (Figure 8). In the rat paw pressure test, metoprine was studied after administration by both i.p. and i.c.v. route (Figure 9). Systemically the doses of 10 and 20 mg kg^{-1} were used; both were significantly antinociceptive and at the highest dose the effect lasted up to 6 h. For i.c.v. administration in rats with permanent cannulae, the doses of 50 and 100 μg per rat were also observed to produce a dose-dependent, long-lasting (6 h) antinociception.

Metoprine effect in the rota-rod test

Metoprine at 20 mg kg^{-1} did not cause any impairment to performance 15 min after i.p. administration. The endurance time on the rotating rod was 282 ± 17 s before and 285 ± 14 s after treatment for control group, and 291 ± 9 s and 297 ± 3 s respectively for metoprine-treated mice (10 animals per group).

Antagonism by (R)-α-methylhistamine (RAMH) of metoprine-induced antinociception

RAMH (20 mg kg^{-1}, i.p.) was able to prevent the antinociception induced by 20 mg kg^{-1}, i.p. of metoprine administered 15 min later in all three tests (Figures 7b, 8, 9a).

In both the rat paw pressure and mouse hot plate tests antinociception was significantly reduced as early as 15 min

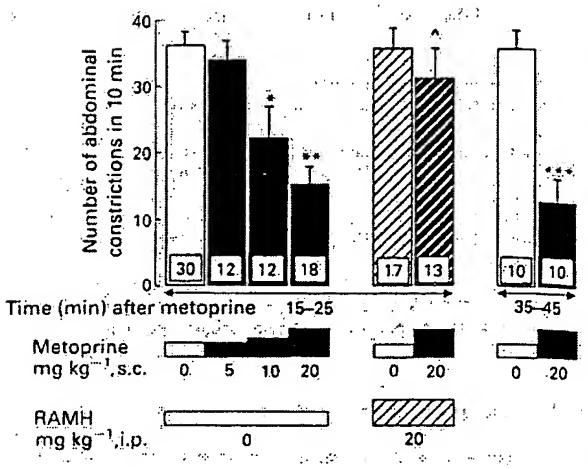

Figure 8 Metoprine antinociception and its antagonism by (R)-α-methylhistamine (RAMH) in the mouse abdominal constriction test. RAMH dihydrogenomaleate was administered 15 min before metoprine. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus controls. $^{\wedge\wedge}P<0.01$ versus metoprine (20 mg kg^{-1})-treated mice. Inside the columns is shown the number of mice.

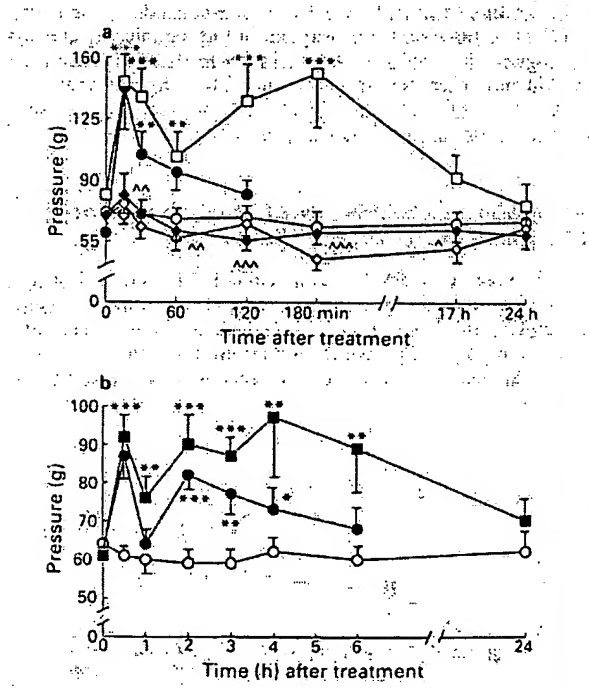

Figure 9 Antinociceptive effect of metoprine in the rat paw pressure test. (a) Effect of i.p. metoprine and its antagonism by (R)-α-methylhistamine (RAMH). Groups (pretreatment i.p. + treatment i.p.) were as follows: saline 0.1 ml $10\,g^{-1}$ + lactic acid 0.3% in saline (○); metoprine 10 mg kg^{-1} (●); saline + metoprine 20 mg kg^{-1} (□); RAMH dihydrogenomaleate 20 mg kg^{-1} + lactic acid 0.3% (◇); RAMH + metoprine 20 mg kg^{-1} (◆). Pretreatment was performed 15 min before treatment. Each point represents the mean of 11–19 rats. (b) Effect of i.c.v. metoprine on rats with permanent cannulae. Groups were (dose per rat i.c.v.): lactic acid 1% in saline 5 μl (○); metoprine 50 μg (●); metoprine 100 μg (■). Each point represents the mean of 7–11 rats. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ versus control group. $^{\wedge}P<0.05$; $^{\wedge\wedge}P<0.01$; $^{\wedge\wedge\wedge}P<0.001$ versus metoprine (20 mg kg^{-1})-treated rats. Vertical lines give s.e.mean.

Table 3 Effect of i.c.v. 1-methylhistamine (MH) on pain threshold in the rat paw-pressure and mouse abdominal constriction tests

Treatment (μg per animal)	Rat paw pressure test Pressure (g) Pretest	30 min after treatment	45 min after treatment	60 min after treatment	Abdom. constr. test Constrictions in 10 min
Saline	59.1 ± 2.0 (6)	58.3 ± 3.0	56.6 ± 3.3	58.3 ± 3.0	28.6 ± 3.3 (19)
MH 0.5	61.6 ± 3.5 (9)	61.1 ± 3.0	62.2 ± 2.2	52.2 ± 2.7	–
MH 1	–	–	–	–	35.5 ± 5.5 (6)
MH 5	–	–	–	–	34.8 ± 4.8 (6)
MH 10	62.2 ± 3.6 (9)	63.3 ± 3.3	58.8 ± 3.8	58.8 ± 2.0	32.7 ± 1.5 (7)
MH 50	–	–	–	–	27.0 ± 3.5 (7)

In parentheses is shown the number of animals

after metoprine treatment, and from 30 min on the antagonism was complete.

The dose of RAMH used did not modify the nociceptive threshold in any of the three tests.

Lack of 1-methylhistamine effect in antinociception

1-Methylhistamine (MH) did not modify the pain threshold in either the mouse abdominal constriction test or the rat paw pressure test (Table 3).

In the abdominal constriction test, MH was studied at a wide range of doses (1–50 μg per mouse, i.c.v.). None of them reduced the number of constrictions induced by acetic acid.

In the paw pressure test also the two doses used (0.5 and 10 μg per rat i.c.v.) were ineffective in modifying the threshold pressure.

Discussion

The present results clearly show that histamine can be either antinociceptive or hyperalgesic depending on the dose. The apparently contradictory effects of this drug might be due to its dose-related action either on the presynaptic receptor or on a postsynaptic receptor, since the selective H_3 receptor antagonist thioperamide caused antinociception, and the selective H_3 receptor agonist, RAMH caused hyperalgesia.

Owing to the reportedly poor ability of histamine to cross the blood-brain barrier (Snyder *et al.*, 1964; Schwartz *et al.*, 1971a), the i.c.v. route of administration was always used. Histamine-induced antinociception, probably due to an action on postsynaptic H_1 or H_2 receptors, was observed in all three tests, showing a dose-dependent relationship. So far, our results confirm previous observations by Glick & Crane (1978), Bhattacharya & Parmar (1985) and Parolaro *et al.* (1989) on rats and Chung *et al.* (1984) and Oluyomi & Hart (1991) on mice. The paw pressure and abdominal constriction seem to be more sensitive tests than the hot plate for detecting histamine antinociceptive effects, as can be noticed from ED_x values. In fact, for the hot plate test, the same cut-off time of 45 s was maintained in order to allow comparisons with the other substances used, but an ED_{30} rather than ED_{50} value needed to be calculated, due to the low antinociceptive effect elicited by histamine. Such discrepancies might be due to the different kinds of noxious stimulus used, i.e. mechanical, chemical and thermal. However, a similar differential sensitivity to analgesic tests is also shared by morphine, some κ-agonists and eseroline (Tyers, 1980; Bartolini *et al.*, 1981b; Yaksh & Noueihed, 1985).

The high reaction scores measured in the three analgesic tests are likely to be due to a real antinociception and not to the loss of ability to react to noxious stimuli, since motor coordination assessed with the rota-rod test was unaffected after the administration of 50 μg per mouse of histamine. The latter dose was preferred to the highest one tested for the rota-rod experiment, since during the hot plate experiments some mice treated with 100 μg i.c.v. developed facial convulsions when held by the tail. Moreover, also Glick & Crane (1978) described catalepsy, besides antinociception, following i.c.v. administration of 200–400 μg per rat of histamine. Further support to histamine acting as a modulator of nociception is given by the experiments of Braga *et al.* (1992). These authors demonstrated that i.c.v. injection of histamine in arthritic rats at the same doses which were antinociceptive in our experiments in the paw pressure test, inhibited the firing of nociceptive thalamic neurones after a noxious stimulus.

Conversely, low doses of histamine were hyperalgesic in the mouse hot plate and rat paw pressure tests. Doses ranging from 50 to 500 fold lower than the analgesic dose in the hot plate and 10 fold lower in the paw pressure test induced a significant decrease in nociceptive threshold (Figures 1a and 2). The dose of 0.5 μg per rat was significantly effective both 30 and 45 min after treatment in the paw pressure test on rats into which histamine was injected during short ether anaesthesia, and the same was adopted for experiments on rats with permanent i.c.v. cannulae, to confirm the data in the absence of ether. This hyperalgesic effect might not seem contradictory when one takes into account the fact that H_3 receptors are 100 fold more sensitive to histamine than the postsynaptic receptors in rat brain slice preparations (Arrang *et al.*, 1983). Thus, hyperalgesia induced by low doses of histamine might be due to a preferential action on H_3 receptors, and, consequently, to inhibition of its own release.

On the other hand, in the mouse abdominal constriction test, histamine-induced hyperalgesia was not detected, as is the case with many other drugs such as arecaidine propargyl ester and CGP 35348, which are hyperalgesic in many tests but inactive in the mouse abdominal constriction test (Bartolini *et al.*, 1992; Malcangio *et al.*, 1991). One reason for this lack might be the abdominal constriction test itself. In fact, as reported by Itoh *et al.* (1989), histamine turnover is enhanced after i.p. injection of 0.7% acetic acid. Thus, if histamine release is enhanced during the abdominal constriction test, the putative hyperalgesic effect of low doses of exogenous histamine is overcome by the augmented amount of endogenous histamine in the brain.

Subsequently, in order to verify our hypothesis that low doses of histamine act at the presynaptic receptor, we studied the effects of both an antagonist and an agonist of the H_3 receptor. Thioperamide, the selective histamine H_3 receptor

antagonist, elicited antinociception in all three tests. It has been reported that parenteral administrations of thioperamide are able to increase histamine turnover (Arrang *et al.*, 1987a; Garbarg *et al.*, 1989; Oishi *et al.*, 1989; Taylor *et al.*, 1992) or release (Itoh *et al.*, 1991) in brain tissues, indicating that it crosses the blood-brain barrier. Thus, our first approach was to study thioperamide effects following parenteral administration. Subsequently, in order to verify whether the site of action of the antinociceptive effect observed with parenteral administration was in the CNS we used the i.c.v. route. Furthermore, for this route we used thioperamide doses (1.1 μg per mouse or rat) which corresponded on a molar basis to a hyperalgesic dose of histamine (0.5 μg per mouse or rat). Despite the lack of any statistically-significant effects of i.c.v.-administered thioperamide in the hot plate test, antinociception was evident in both abdominal constriction and paw pressure tests for both administration routes. This is in agreement with the reported action of thioperamide as a histamine H_3 receptor antagonist, and the antinociception observed might well be due to blockade of the H_3 receptor and, consequently, to increased endogenous histamine release. This mechanism of action is not surprising if one bears in mind that two postsynaptic antagonists of the opioid and cholinergic systems naloxone and atropine, have also been demonstrated to induce antinociception when administered at low doses which are ineffective on the postsynaptic site, but which are capable of blocking the respective presynaptic receptors (Levine *et al.*, 1979; Ghelardini *et al.*, 1990).

In the abdominal constriction and hot plate tests it was also observed that the antinociceptive effect diminished with increasing doses of thioperamide. Since thioperamide has been reported to have a K_i of 4 nM on the H_3 receptor and >10,000 nM on the H_1 or H_2 receptors (Schwartz *et al.*, 1990), the hypothesis of a postsynaptic antagonism can be ruled out. Instead, competition on the H_3 receptor between thioperamide and the endogenous histamine released might be postulated. When the dose of thioperamide is sufficiently strong, the amount of histamine released is high enough to compete with thioperamide, thus activating the mechanism of negative feedback on the release and, consequently, antagonizing thioperamide antinociceptive effect. This hypothesis might also explain the low maximum effect of thioperamide observed by us.

As for the histamine H_3 agonist, RAMH, hyperalgesia was observed at 3 μg per mouse or 1 μg per rat i.c.v. as soon as 15 min after treatment. In order to assess whether a quick hyperalgesia is also obtainable following systemic administration, a dose as high as 100 mg kg^{-1} was used in the hot plate test, since 20 mg kg^{-1}, i.p. had no effect within 1 h of treatment. Such a rapid effect is probably due to inhibition of histamine release. Conversely, the delayed hyperalgesia observed in the rat paw pressure test following the injection of 20 mg kg^{-1} i.p. (from 4 to 7 h after treatment) might be due to histamine synthesis inhibition. Garbarg *et al.* (1989) in fact reported an increase in synaptosomal histamine and a decrease in N^{τ}-methylhistamine levels which reached statistical significance 3 h after oral administration of 10 mg kg^{-1} of RAMH (expressed as the base), and a reduction in cortical [^{3}H]-histamine synthesis which was already significant 30 min after treatment. All these effects lasted up to 6 h. Thus, it might be postulated that the hyperalgesia observed in our conditions is due to a dose-dependent effect of RAMH on endogenous histamine release. Moreover, hyperalgesia induced by stimulation of autoreceptors is not unusual: another example is in the cholinergic system, where the presynaptic agonist, arecaidine is able to lower the nociceptive threshold in both hot plate and paw pressure tests (Bartolini *et al.*, 1992).

Pretreatment with an i.p. dose of RAMH which did not modify the pain threshold in mice completely prevented the antinociception induced by 20 mg kg^{-1} of thioperamide in both abdominal constriction and hot plate tests. This antagonism might reflect the action of both substances on the same receptor, since for both of them a high selectivity on the histamine H_3 receptor has been reported (Arrang *et al.*, 1987b; Garbarg *et al.*, 1989). In the rat paw pressure test a similar antagonism was also observed following i.c.v. administration. Although 1 μg per rat of RAMH had an hyperalgesic effect, the antagonism is probably not due to a simple summing of the effects of the two substances, since the values obtained with double treatment are much lower than those resulting from the subtraction of the hyperalgesic effect of RAMH from the analgesic effect of thioperamide. On the other hand, the dose of 10 μg per rat of thioperamide was preferred as eliciting a clearer and longer-lasting antinociception, while for RAMH the dose of 1 μg per rat was chosen as about a ten fold lower dose (on a molar basis) than the thioperamide one. The choice was made taking into account that for RAMH and thioperamide a 1:8 affinity ratio has been reported for the H_3 receptor (Taylor *et al.*, 1992). Conversely, when i.p. or s.c. administration was performed (i.e. in experiments on mice), two equal doses were adopted for the two substances, since, despite its higher affinity for the H_3 receptor, RAMH seems to have even more difficulty in crossing the blood-brain barrier than thioperamide (Taylor *et al.*, 1992).

The histaminergic system in antinociception was further studied by altering histamine brain levels. The choice of histidine as a tool for studying the role of endogenous histamine in antinociception was made on the basis that, as Schwartz *et al.* (1972) and Abou *et al.* (1973) demonstrated, its decarboxylating enzyme is not saturated in normal conditions. Systemic administrations of L-histidine are therefore able to enhance brain histamine levels.

In our experiments on rats, L-histidine induced antinociception with both doses used, with a maximum effect at 2–3 h after treatment. This time-course reflects that of endogenous brain histamine levels observed in rats by Schwartz *et al.* (1972) and Sheiner *et al.* (1985) after i.p. administration of L-histidine at the same doses; brain histidine levels were maximal within 1 h of a loading of 500 mg kg^{-1}, i.p., halved at 1.5 h and constantly decreased thereafter, while histamine levels reached peak values within 2–3 h of injection. This seems to support the hypothesis that antinociception is due to histamine deriving from the conversion of L-histidine, and not to histidine itself.

In mice, too, antinociception takes place slowly after L-histidine administration. In the abdominal constriction test the maximal inhibition of stretching movements was observed 2 h after treatment, while in the hot plate test antinociception reached a plateau from 1 to 2.5 h for the highest dose; in both tests the effect diminished 3 h after histidine injection. As seems also to be the case with rats, the antinociception in mice occurs parallel to, though later than, the alterations in brain histamine levels observed after a single (850 and 1000 mg kg^{-1}) or multiple (3 × 1000 mg kg^{-1}) i.p. L-histidine injection (Taylor & Sneider, 1972; Cosentin *et al.*, 1974).

With L-histidine as with histamine, the mouse hot plate is less sensitive than the mouse abdominal constriction and rat paw pressure tests. In fact, in the hot plate test, statistically-significant antinociception was found at the doses of 500 and 1500 mg kg^{-1}, i.p., although the effect was very small. The latter dose might seem to be high, but it should be considered that another amino acid, L-DOPA, also needs to be administered at a dose of 1000 mg kg^{-1} in order to show any effect (Blaschko & Chrusciel, 1960). Nevertheless, even at the highest dose of L-histidine, the gross behaviour of animals was not modified during our experiments, as formerly reported by Schwartz *et al.* (1972) for rats and by Abou *et al.* (1973) for rabbits.

Further evidence of the fact that L-histidine-induced antinociception is due to its conversion into histamine is given by our results obtained with FMH. This substance, developed by Kollonitsch *et al.* (1978) as a highly selective irreversible

HDC inhibitor, has been demonstraed to halve brain histamine levels in mice within 1 h, up to 24 h (Garbarg *et al.*, 1980; Maeyama *et al.*, 1982; 1983), and in rats (Oishi *et al.*, 1984). The brain histamine levels which are reduced by FMH are likely to be those of neural cells, since in mutant mice devoid of mast cells brain histamine is almost totally abolished by FMH (Maeyama *et al.*, 1983). Thus, total prevention by FMH of L-histidine-induced antinociception at all the times considered was probably due to blockade of neuronal HDC activity.

The present findings partly contradict those of Oluyomi & Hart (1991), who observed histidine-induced antinociception only in the mouse hot plate test. Moreover, this effect was already present 15 min after histidine administration (400 mg kg^{-1}, i.p.). The authors attributed the lack of effect in the mouse abdominal constriction test to a lack of action on peripheral histamine receptors.

Our further approach to the study of endogenous histamine effects in antinociception was to raise brain histamine levels by inhibiting its catabolism. Since brain histamine catabolism occurs almost exclusively via ring methylation (Schwartz *et al.*, 1971b; Schayer & Reilly, 1973), we used for this purpose metoprine, an antifolate which has been described as a highly potent, competitive HMT inhibitor devoid of any action on HDC (Duch *et al.*, 1978) and which is able to enhance histamine release in *in vivo* microdialysis studies following i.p. administration (Itoh *et al.*, 1991).

Due to its high liposolubility (Duch *et al.*, 1978), in the present experiments metoprine was usually administered i.p. in mice and rats to obtain a more general distribution of the substance within the central nervous system. However, in some experiments with the paw pressure test, metoprine was also given i.c.v. through permanent cannulae, in order to make sure that its site of action was in fact the CNS. In both cases and species the dose-dependence of the antinociceptive action of metoprine seems to reflect the degree of inhibition of brain HMT activity. Hough *et al.* (1986) reported that 5 mg kg^{-1}, i.p. of metoprine caused a reduction of 70% in whole brain HMT activity, while a 90% reduction can be obtained with 20–30 mg kg^{-1}. The present data seem to suggest that a 70% reduction is not sufficient to induce antinociception, since at least 10 mg kg^{-1} were found to be necessary to obtain a statistically significant rise in the nociceptive threshold (abdominal constriction test). A single dose (20 mg kg^{-1}, i.p.) was found to induce an antinociception whose potency was comparable to that induced by morphine (9 mg kg^{-1}, s.c. in the mouse hot plate test and 5 mg kg^{-1}, s.c. in the rat paw pressure test 15 and 30 min after treatment respectively). At the same dose metoprine-treated mice were able to stay balanced on the rotating rod, while morphine given at doses higher than 8.2 mg kg^{-1}, s.c. significantly impaired animal performance (data not shown).

Furthermore, metoprine antinociception was also longer-lasting than that of morphine; this is consistent with the reported metoprine half-life of 17 h (Duch *et al.*, 1978). In fact, a long-lasting antinociceptive effect is also shared by other neurotransmitter catabolism inhibitors, such as the enkephalinase inhibitors, thiorphan (Greenberg & O'Keefe, 1982), D-phenylalanine (Ehrenpreis *et al.*, 1983), azidothiorphan (Beaumont *et al.*, 1987) and SCH 34826 (Chipkin *et al.*, 1988), the GABA transaminase inhibitors amino-oxyacetic acid (Bartolini *et al.*, 1981a; Sawynok & LaBella, 1982) γ-acetylenic GABA and γ-vinyl GABA (Buckett, 1980; Sawynok & LaBella, 1982), and the cholinesterase inhibitor diisopropylfluorophosphate (DFP) (Costa & Murphy, 1986; Green & Kitchen, 1986).

To assess whether metoprine-induced antinociception was due to the blockade of HMT, animals were pretreated with RAMH. The complete antagonism observed is consistent with the results of Oishi *et al.* (1989), who reported a decrease in the levels of the catabolism product 1 h after RAMH treatment. Thus, since inhibition of histamine release by RAMH prevents metoprine-induced antinociception, it can be argued that the metoprine antinociceptive effect might be due to endogenous histamine which has not been catabolized.

The effect of MH was studied in the two tests most sensitive to histamine, the rat paw pressure and mouse abdominal constriction tests, at the same doses at which histamine was antinociceptive. As expected, the catabolism product was ineffective. Schwartz *et al.* (1971b) reported that 500 μg per rat intracisternally is able to inhibit HMT, but in their experiments the doses used were at least 10 fold higher than in ours.

In conclusion, our data taken together indicate that endogenous histamine is an antinociceptive neurotransmitter. All the substances used in the present work had an effect which was consistent with their reported ability to modulate endogenous histamine release or alter brain histamine levels. Thus when histamine brain levels or release are increased, antinociception can be observed, conversely, when the amount of histamine released is decreased, hyperalgesia occurs. It thus seems likely that the histaminergic system, like many other neuronal systems, plays an important role in the modulation of central perception of nociceptive stimuli.

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Short communication

Histaminergic neurons facilitate social memory in rats

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Abstract

The social memory test was used so as to investigate whether brain histamine is involved in short-term memory. Histamine injected intracerebroventricularly (i.c.v.) decreased investigation time of a juvenile rat by an adult rat. A similar effect was elicited by i.c.v. administration of histidine. Compared with the control animals, rat pretreatment with α-fluoromethylhistidine (FMH), which inhibits neuronal synthesis of histamine, prolonged recognition time. The H_3-receptor agonist immepip also prolonged investigation time, while the H_3-antagonist thioperamide exerted the opposite effect. Treatment with histidine increased, while treatment with FMH decreased histamine levels in various brain regions. It is concluded that histamine released from histaminergic neurons facilitates short-term memory.

Keywords: Short-term social memory; Histamine; α-Fluoromethylhistidine; Immepip; Thioperamide; Histidine

The importance of histamine for brain function has been extensively investigated in recent years. Concerning long-term memory, it has been reported that histamine facilitates memory retrieval in rats subjected to passive avoidance procedures [1,6–9]. Moreover, it has been found that histamine improves [14] the scopolamine-induced memory deficit [5,11]. These effects of histamine are mediated by H_1-receptors [8,9,11]. The aim of our investigation was to prove whether histaminergic neurons of the brain are also implicated in short-term memory. For this purpose, we used the olfactory, social memory test which is based on the recognition of a juvenile rat by a male adult, sexually-experienced rat [2,15,16].

Male Sprague–Dawley rats, approximately 6 months old and weighing 400–500 g, were used as adult rats, and male Sprague–Dawley rats, 1 month old and weighing 80–120 g, as juvenile rats. All animals were housed under standard laboratory conditions with food and water ad libitum. Experiments were carried out each day between 1100 and 1800 h. For intracerebroventricular (i.c.v.) injections of drugs, a guide cannula (outer diameter: 0.65 mm, inner diameter: 0.4 mm) with its stylet was stereotaxically inserted in the right lateral ventricle of the rat anaesthetized with pentobarbital sodium (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.). The coordinates were (mm): AP −1.3, L 1.7, V 3.8 [12]. After a recovery period of four days, an adult rat was placed for 10 min in the observation cage. A 5 min-exposure to a juvenile followed and the time period, the adult rat investigated the juvenile rat was recorded. Immediately after the first contact, drugs were applied by i.c.v. injection. The second exposure followed 45 min (histamine, histidine or thioperamide), 30 min (immepip), or 155 min (α-fluoromethylhistidine, FMH) after drug administration (see below), and time necessary for recognition was recorded as mentioned above. Data were calculated as the ratio "recognition duration during second contact/investigation duration during first contact". This transformation was carried out so as to minimize day-to-day variations in rat behaviour and to equalize individual differences between animals. Preliminary experiments have shown that when intervals between first and second contact were shorter than 30 min, the duration of recognition during second contact was reduced (RID approximately 0.7). This did not occur when intervals were longer than 45 min (RID approximately 1). For this reason, effects of drugs facilitating memory were investigated after an interval of 45 min, while effects of drugs suppressing memory were studied after an interval of 30 min, i.e. when the old rat still recognized the juvenile. Drugs were diluted in artificial cerebrospinal fluid (CSF) and 10 μl were injected over a period of 30 s through a stainless steel cannula (outer diameter: 0.38 mm,

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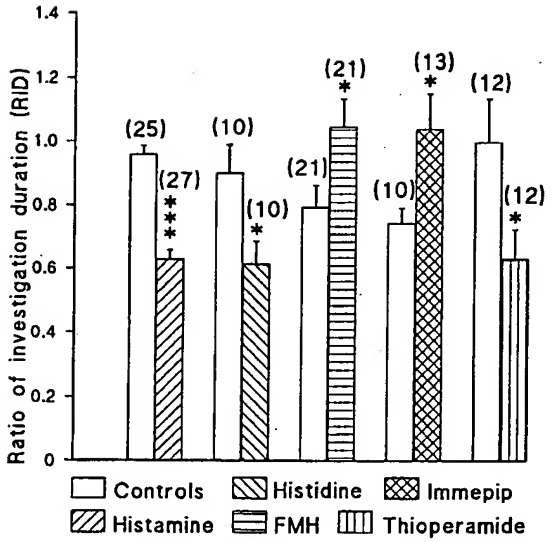

Fig. 1. Effects of histamine (200 ng), histidine (37 μg), FMH (80 μg), immepip (0.5 μg) and thioperamide (0.7 μg) on the olfactory social memory test. Scale: ratio of recognition duration during the second contact to that during the first contact (RID). Means ± S.E.M., number of experiments in parentheses. * $P < 0.05$, *** $P < 0.001$.

inner diameter 0.2 mm) which was inserted in the guide cannula. The injection cannula was connected by a tubing to a syringe pump. Doses of histamine dihydrochloride, histidine hydrochloride monohydrate, thioperamide maleate, FMH monohydrochloride and immepip dihydrobromide, refer to the corresponding bases. In separate experiments, histamine levels in the brain were determined 45 and 120 min after i.c.v. injections of histidine and FMH, respectively, rats were killed by decapitation, the brains quickly removed, placed on ice and dissected into three sections [4]: frontal cortex, hypothalamus and the cannulated, right hemisphere of the rest of brain. Tissue was weighed and homogenized by sonication in 3 ml (frontal cortex and rest of brain) or 0.5 ml (hypothalamus) of 0.1 M perchloric acid containing 0.1 mM Na_2 EDTA. The homogenates were centrifuged for 10 min at 10 000 × *g*. Histamine was determined in the supernatant by HPLC combined with fluorimetric detection after derivatization with *o*-phthaldialdehyde [13].

During first contact, the investigation period of the juvenile rat by the adult rat was very similar in the 10 groups of animals and amounted to 96 ± 3 s (mean values ± S.E.M., $n = 10$). Injection of histamine (200 ng) led to a significant decrease in recognition time during the second contact (Fig. 1). Similarly, short-term memory was facilitated by the treatment with 37 μg of the histamine precursor histidine. Compared with the control animals, on the other hand, pretreatment with 80 μg FMH prolonged investigation time. FMH inhibits the synthesis of histamine [3], thus depleting neuronal sites without influencing mast cell histamine levels [3,10,18]. The findings indicate that elevated histamine concentration in the brain facilitates short-term memory, while depletion of neuronal histamine suppresses memory in the rat. Determination of histamine levels in the brain confirmed effects of the used compounds on endogenous histamine at the time of test performance; pretreatment with histidine led to a pronounced increase in histamine concentrations in the brain, particularly in the hypothalamus. On the other hand, histamine levels were drastically diminished by FMH (Table 1).

To further investigate the importance of histaminergic neurons of the brain for memory, the H_3-receptor agonist immepip and the H_3-antagonist thioperamide were applied by i.c.v. injection. In contrast to histamine, pretreatment with the agonist immepip [17] (0.5 μg) prolonged recognition time, while the antagonist thioperamide (0.7 μg) elicited the opposite effect (Fig. 1). These findings suggest that, by acting on presynaptically and/or postsynaptically located H_3-receptors, immepip and thioperamide inhibit and enhance the release of neuronal histamine, respectively, thus modulating short-term memory. It remains to be clarified, whether H_1- and/or H_2-receptors are involved in the facilitation of short-term memory by histamine.

In conclusion, our findings show that brain histamine decreases recognition time in the olfactory social memory test. Hence, histaminergic neurons in the brain seem to facilitate short-term memory.

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Table 1
Effects of centrally applied histidine and FMH on histamine levels in various brain areas

Treatment	Hypothalamus	Frontal cortex	Rest of brain
		Histamine (ng/g tissue)	
Controls	157.95 ± 31.14 (3)	25.30 ± 3.35 (4)	42.55 ± 9.39 (4)
Histidne	683.24 ± 73.00 ** (4)	56.45 ± 9.72 * (4)	68.77 ± 3.50 * (4)
Controls	147.33 ± 12.36 (3)	56.42 ± 7.58 (4)	46.18 ± 5.33 (4)
FMH	69.00 ± 0.41 ** (4)	28.67 ± 3.63 * (4)	18.70 ± 3.50 ** (4)

Histidine was injected 45 min, FMH 155 min prior to decapitation. Control animals were treated with CSF. Number of experiments in parentheses. Means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test for grouped data).

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Short communication

Effects of histamine on MK-801-induced memory deficits in radial maze performance in rats

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Abstract

The effects of histamine on the spatial memory deficits induced by MK-801 were investigated using the eight-arm radial maze paradigm in rats. Intracerebroventricular (i.c.v.) injection of histamine or thioperamide, and intraperitoneal (i.p.) injection of histidine improved the spatial memory deficits induced by MK-801. Similar results were obtained with 2-thiazolylethylamine. In contrast, 4-methylhistamine showed no significant effect. Based on these observations, it seems likely that the protective effect of histamine on MK-801-induced spatial memory deficit is mediated by H_1-receptors. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: MK-801; Histamine; H_1-receptor; H_3-receptor; Radial maze performance

It has been reported that *N*-methyl-D-aspartate (NMDA) receptors, a subclass of glutamate receptors, play an important role in learning and memory [7,9]. In addition, the NMDA receptor antagonist MK-801 was shown to impair various kinds of learning behavior such as passive avoidance response, radial maze performance and Morris maze performance in rats [4,8,21].

Behavioral studies have indicated that histamine results in facilitation of the impaired memory retrieval induced by aging or hippocampal lesions in rats using passive and active avoidance tests [5,10,13]. On the other hand, depletion of hippocampal histamine content was shown to adversely affect recall in rats in both active avoidance tasks and radial maze performance [6,12]. Recently, the relationship between histaminergic neurons and NMDA receptors has attracted a great deal of attention [1,3,17,20]. In cultured hippocampal neurons, histamine was shown to modulate NMDA receptor function mediating synaptic transmission [1,20]. However, the interactions between NMDA receptors and histaminergic receptors in cognition processes have not been elucidated. The aim of the present study was to clarify whether histamine is involved in the spatial memory deficit induced by MK-801 using the eight-arm radial maze task in rats.

Male Wistar rats weighing 200–280 g (Charles River, Tokyo, Japan), were maintained in an air-conditioned room with controlled temperature (22–26°C) and humidity (40–70%). They were housed in individual cages with a 12 h light–dark cycle (lights on from 0800–2000 h). Prior to behavioral tests, the body weight of rats ($n = 58$) was gradually reduced over a period of 1 week to 80–85% of their free-feeding weight, and then animals were kept on a restricted diet for the rest of the experiment. Water was given ad libitum. Rats were anesthetized with sodium pentobarbital (35 mg/kg, i.p., Abbot Laboratories, North Chicago, IL, USA), fixed on a stereotaxic apparatus (Narishige, SR-5, Tokyo, Japan), and a guide cannula made of stainless steel tubing 700 μm in outer diameter was implanted into the right lateral ventricle according to the following coordinates measured from bregma [16]; AP: −0.9 mm, L: 1.5 mm, H: 3.8 mm from the skull. At least 14 days were allowed for recovery from surgery.

The experimental apparatus and the procedures were described in our previous report [6]. Briefly, to familiarize the rats with the radial maze prior to training they received one daily habituation trial for two days. After adaptation, all rats were trained with one trial per day. In each trial, a single food pellet (45 mg each, Bio-Serv, Frenchtown, NJ, USA) was placed in the food cup in each of the eight arms. The rat was placed in the center of platform and allowed to make arm choice to obtain food pellets until all eight pellets had been eaten or 10 min had elapsed. Rats were

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trained continually until reaching a criterion of at least seven different arms in the first eight choices and all eight within the first nine choices before the test. The animals were tested with either drug or vehicle after successfully completing the maze on three consecutive days. The test trial was performed for 3 min or until the rat collected all pellets. The following indices of maze performance were used to represent accurate choice: (1) number of total errors (TE); (2) number of initial correct response (ICR).

Histamine dihydrochloride (Wako, Osaka, Japan), 2-thiazolylethylamine dihydrochloride (SmithKline Beecham, London, UK), 4-methylhistamine dihydrochloride (SmithKline Beecham, London, UK) and thioperamide hydrochloride (provided by Eisai, Tokyo, Japan) were dissolved in saline and injected i.c.v. in a fixed volume of 5 μl over a period of 60 s at a constant speed with a continuous infusion pump (KN-201, Natsume, Tokyo, Japan). (+)-MK-801 hydrogen maleate (Funakoshi, Tokyo, Japan), pyrilamine maleate (Sigma, St. Louis, MO, USA), zolantidine (a gift from SmithKline Beecham., London, UK) and histidine monohydrochloride (Wako, Osaka, Japan) dissolved in saline, were injected i.p. All procedures involving animals were conducted in accordance with the guidelines of the Animal Care and Use Committee, Faculty of Pharmaceutical Sciences, Okayama University.

One-way Analysis of Variance with Dunnett's test was used to test for statistical significance of the TE and ICR in the radial maze, and a difference of $p < 0.05$ was regarded as statistically significant.

Table 1 shows the effects of histamine (i.c.v.) and histidine (i.p.) on radial maze performance deficits induced by MK-801 (0.1 mg/kg). MK-801 produced a marked increase in the number of TE, and a decrease in the number of ICR. The i.c.v. injection of histamine antagonized the effect of MK-801 in a dose-dependent manner; no significant effect was observed at a dose of 50 ng, while at a dose of 100 ng a significant increase was observed in the number of ICR ($p < 0.05$), and at a dose of 200 and 500 ng significant decreases were observed in the number of TE and the numbers of ICR were increased ($p < 0.05$). Similar results were obtained with histidine; i.e., significant effects were observed at doses of 200 or 500 mg/kg ($p < 0.05$).

Table 1
Effects of histamine and histidine on memory deficits induced by MK-801
Histamine was injected i.c.v. 20 min after MK-801 injection, and histidine was injected i.p. 2.5 h before MK-801 injection. Each value represents the mean ± S.E.M. of 22–28 rats. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with MK-801 + saline-treated group.

Drug	Dose	Total errors	Initial correct response
Saline	–	0.2 ± 0.1	7.8 ± 0.1
MK-801 +	0.1 mg/kg		
Saline	–	3.8 ± 0.9	5.4 ± 0.4
MK-801 +	(0.1 mg/kg)		
Histamine	50 ng	2.6 ± 0.8	6.1 ± 0.4
	100 ng	1.7 ± 0.6	6.8 ± 0.4*
	200 ng	1.0 ± 0.5*	7.3 ± 0.3**
	500 ng	0.8 ± 0.4*	7.2 ± 0.3**
MK-801 +	(0.1 mg/kg)		
Histidine	200 mg/kg	2.1 ± 0.8	6.7 ± 0.3*
	500 mg/kg	1.1 ± 0.5*	7.0 ± 0.3**
	1000 mg/kg	0.6 ± 0.3**	7.2 ± 0.3**

Table 2
Effects of H_1- and H_2-agonists on memory deficits induced by MK-801
2-Thiazolylethylamine and 4-methylhistamine were injected i.c.v. 20 min after MK-801 injection. Each value represents the mean ± S.E.M. of 18–26 rats. $^{*}p < 0.05$ as compared with MK-801 + saline-treated group.

Drug	Dose	Total errors	Initial correct response
MK-801 +	0.1 mg/kg		
Saline	–	3.9 ± 1.0	5.3 ± 0.5
MK-801 +	(0.1 mg/kg)		
2-Thiazolylethylamine	100 ng	2.1 ± 0.6	6.3 ± 0.4
	200 ng	1.5 ± 0.5*	6.8 ± 0.4*
	500 ng	1.4 ± 0.4*	7.0 ± 0.3*
MK-801 +	(0.1 mg/kg)		
4-Methylhistamine	100 ng	3.2 ± 0.8	6.0 ± 0.5
	200 ng	2.5 ± 0.6	6.4 ± 0.4
	500 ng	1.9 ± 0.6	6.6 ± 0.4

The i.c.v. injection of 2-thiazolylethylamine, dose-dependently antagonized the spatial memory deficits induced by MK-801 (Table 2). Significant effects were observed in both parameters at doses of 200 and 500 ng ($p < 0.05$). On the other hand, 4-methylhistamine showed no significant effect on memory deficits induced by MK-801 even at a dose of 500 ng. In contrast, thioperamide significantly antagonized MK-801-induced spatial memory deficits at a dose of 50 μg ($p < 0.05$)(Fig. 1). Pyrilamine, a representative H_1-receptor antagonist, antagonized the action of histidine improving MK-801-induced memory impairment (Table 3). A significant effect was obtained at a dose of 5 mg/kg ($p < 0.05$). However, zolantidine, a representative H_2-receptor antagonist, showed no marked effect on histidine action even at a dose of 20 mg/kg.

In the present study, i.c.v. injection of histamine or i.p. injection of histidine improved the impairment of radial maze performance induced by MK-801 (0.1 mg/kg), at a dose which was previously reported to impair memory in radial maze performance [4,15]. On the other hand, running time per choice was not influenced by drug treatments (data not shown). Therefore, the ameliorating effect of histamine on the spatial cognition deficit induced by MK-801 appeared to be essentially unrelated to changes in locomotor activity.

Recently, it was reported that histamine facilitates NMDA-mediated excitatory transmission in the hippocampus and induction of long-term potentiation in the CA1 region of the hippocampus [2,3]. In addition, Rodriguez et

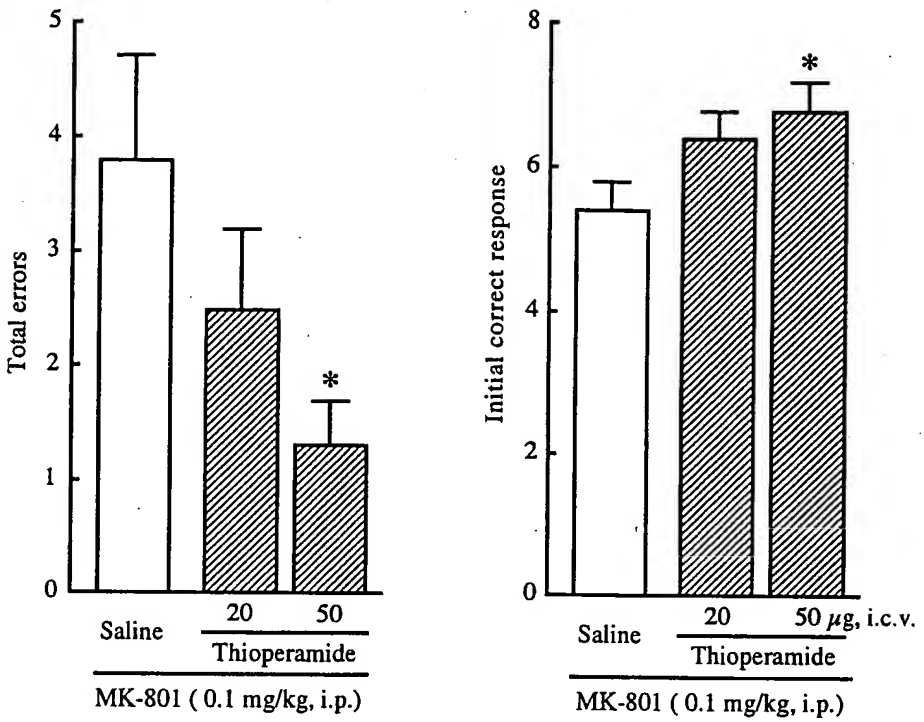

Fig. 1. Effects of thioperamide on memory deficits induced by MK-801 (0.1 mg/kg) on eight-arm radial maze performance in rats. MK-801 was injected i.p. 30 min before test trial, and thioperamide was administered i.c.v. 15 min after MK-801 injection. Each value represents the mean ± S.E.M. of 20–28 rats. $^*p < 0.05$ as compared with saline-treated control group.

al. [17] also reported that histaminergic fibers arriving at the hippocampus could be involved in the modulation of glutamatergic transmission. Also, they found that histamine produced a concentration-dependent increase in glutamate release in the hippocampus via H_1 and H_2 receptors [17]. Therefore, the glutamate released from the hippocampus by histamine may be responsible for the observed improvement of the MK-801-induced spatial memory deficits.

It is reported that activation of dopamine neurotransmission contributes to memory deficit induced by MK-801 in both spatial delayed alternation performance and one-trial inhibitory avoidance response [14,19]. Schlicker et al. [18] found that histamine inhibits dopamine release in the mouse brain via presynaptic H_3-receptors. Therefore, it is reasonable to presume that protective effect of histamine on MK-801-induced memory deficits may, at least in part, be related to dopaminergic mechanisms.

It has also been reported that postsynaptic H_1-receptors and presynaptic H_3-receptors are important for learning and memory in both active avoidance responses and radial maze performance in rats [6,11]. In the present study, 2-thiazolylethylamine, a representative H_1-receptor agonist, improved the memory deficit induced by MK-801. In contrast, no effect was observed with 4-methylhistamine, a representative H_2-receptor agonist. Similarly to histamine, thioperamide, a representative H_3-receptor antagonist improved the MK-801-induced spatial memory deficit in radial maze performance. On the other hand, the action of histamine improving MK-801-induced memory deficit was antagonized by pyrilamine, but not by zolantidine. Therefore, these findings suggested that the antagonizing effect of histamine on MK-801-induced memory deficit may be mediated by postsynaptic H_1-receptors and presynaptic H_3-receptors.

Table 3
Effects of pyrilamine and zolantidine on histidine-induced amelioration of memory deficit induced by MK-801
Pyrilamine and zolantidine were simultaneously injected with MK-801, and histidine was injected 2.5 h before MK-801 injection. Each value represents the mean ± S.E.M. of 17–25 rats. $^*p < 0.05$ as compared with MK-801 + histidine + saline-treated group.

Drug	Dose (mg/kg)	Total errors	Initial correct response
MK-801 + Histidine + Saline +	0.1 1000 –	0.9 ± 0.3	7.3 ± 0.3
MK-801 + Histidine + Pyrilamine	0.1 1000 1	1.2 ± 0.5	7.0 ± 0.4
	2	2.5 ± 0.8	6.9 ± 0.4
	5	3.8 ± 1.1*	6.0 ± 0.5*
MK-801 + Histidine + Zolantidine	20	2.9 ± 0.8	6.8 ± 0.4

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CIAL REPORT

teraction of clozapine with the histamine H_3 receptor in rat rain

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We examined possible interactions between neuroleptics and the histamine H_3 receptor and found an interaction of clozapine with this receptor. In competition binding experiments, using the H_3 antagonist, [^{125}I]-iodophenpropit, we observed a K_i of 236 ± 87 nM. Functionally, clozapine was studied on the H_3-mediated inhibition of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) release from rat brain cortex slices. Clozapine acts as an antagonist with an apparent K_B value of 79.5 nM.

Keywords: Histamine H_3 receptor; clozapine; neuroleptic; rat brain slices; [^{125}I]-iodophenpropit; [^{3}H]-5-HT release

Introduction Recent studies suggest that histamine may be involved in schizophrenia. In treatment-resistant patients, the mean level of *tele*-methylhistamine (t-MH) in cerebrospinal fluid, an index of histaminergic activity, was 2.6 fold higher than in controls (Prell *et al.*, 1995). Cerebrospinal fluid levels of t-MH correlated positively with those of other neurotransmitters metabolites, and with severity of schizophrenic symptoms (Prell *et al.*, 1995). Consonant with increased histaminergic activity are a reduction in densities of H_1 receptors in *postmortem* frontal cortex (Nakai *et al.*, 1991) and the reported therapeutic actions of famotidine, an H_2 antagonist in schizophrenic patients (Kaminski *et al.*, 1990; Deutsch *et al.*, 1993). Evidence for elevated histaminergic activity suggests possible functional anomalies in H_3 receptors. H_3 autoreceptors regulate release of histamine as well as other neurotransmitters (H_3 heteroreceptors), that may be involved in schizophrenia (e.g. 5-hydroxytryptamine (5-HT) and dopamine) (Fink *et al.*, 1990). Therefore, we examined the effects of neuroleptics on H_3 receptors, giving particular attention to clozapine.

Methods *Binding assays* Competition binding experiments were performed according to the method fully described by Jansen *et al.* (1994).

Functional assays (Based on Van der Werf *et al.*, 1987). [^{3}H]-5-hydroxytryptamine (5 μCi, 11 Ci mmol^{-1}) in 2 ml Krebs Ringer buffer was used to preload the rat neocortex slices. Loaded slices were perfused at 0.3 ml min^{-1}. Six 10 min fractions were collected, tritium overflow was evoked by electrical stimulation (rectangular pulses, 30 mA and 2 ms, 1 Hz) during the second fraction. Lastly, the slices were perfused with 0.1 N HCl to extract the remaining tritium in the tissue.

Results *Binding studies* Displacement of 0.25 nM [^{125}I]-iodophenpropit binding by clozapine (Figure 1a) fitted best to a one site model ($P<0.05$) using the non-linear curve fitting programme LIGAND, resulting in a K_i value of 236 ± 87 nM ($n=6$, mean ± s.e.mean). Fitting data to a sigmoidal curve yielded a slope equal to 0.80 ± 0.05. Other neuroleptics studied showed relatively low affinity for [^{125}I]-iodophenpropit binding (Table 1).

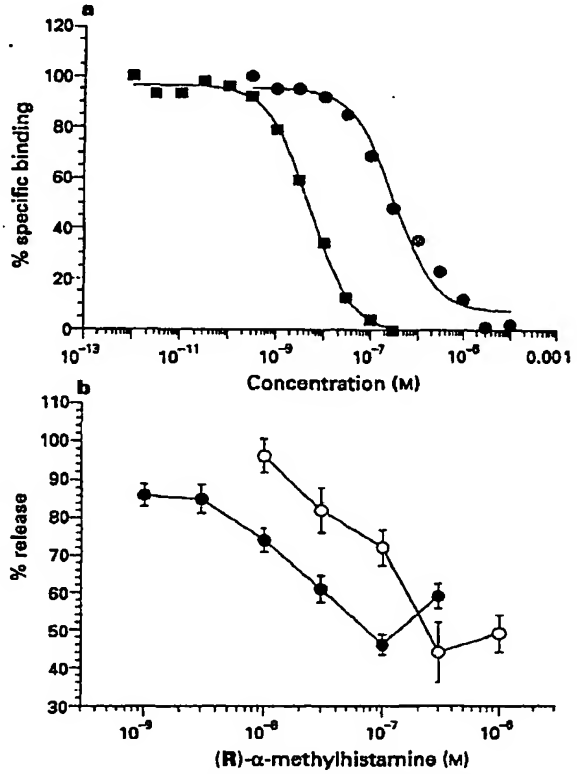

Figure 1 (a) Inhibition of [^{125}I]-iodophenpropit binding (0.25 nM) by the H_3 receptor antagonist, thioperamide (■) and clozapine (●). Data are expressed as % specific binding, determined using 0.3 μM thioperamide and represented 55%–65% of the total binding. The curves show one site fittings of a single representative experiment ($n=6$) with triplicate determinations. (b) Inhibition by (R)-α-methylhistamine of the electrically stimulated [^{3}H]-5-HT release from rat neocortex slices in the absence (●) and presence (○) of 1 μM clozapine. Each point represents mean ± s.e.mean of three or four different experiments performed in duplicate. The EC_{50} values are determined from the mean values according to the equation $E = 100 - (100 - E_{max})/(1 + (EC_{50}/[A])$ where E represents the effect observed at any agonist concentration of [A], EC_{50} represents the agonist concentration that produces half-maximal effect, and E_{max} the maximal effect. The dissociation constant (K_B) was calculated from the equation $[A']/[A] = 1 + [B]/K_B$, where [A] is the concentration of the agonist that produces half-maximal effect, [A'] is the concentration of the agonist that produces half-maximal effect in the presence of the antagonist concentration [B].

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Table 1 Affinity of H_3 receptor agonists and antagonists and of several neuroleptics [RBI] for [^{125}I]-iodophenpropit binding sites to rat cortex compared with their functional potency (apparent values of K_B and K_i are shown, based on competitive antagonism)

Ligand	Inhibition of [^{3}H]-5-HT release	[^{125}I]-iodophenpropit binding
Histamine	EC_{50} = 48.2 nM (3)	K_{iH} = 38 ± 10 nM (4)[a]
(R)-α-methylhistamine	EC_{50} = 6.0 nM (4)	K_{iH} = 3.5 ± 1.2 nM (4)[a]
Thioperamide	K_B = 1.1 nM (3)	K_i = 4.3 ± 1.6 nM (7)[a]
Impromidine	K_B = 19.5 nM (3)	K_i = 51 ± 9 nM (3)[a]
Clozapine	K_B = 79.5 nM (4)	K_i = 236 ± 87 (6)
Chlorpromazine	ND	IC_{50} > 10 μM (2)
Haloperidol	ND	IC_{50} > 10 μM (2)
Spiperone	ND	IC_{50} > 10 μM (2)

For the binding studies data shown are mean ± s.e.mean. The values in parentheses represent the number of independent experiments. Functional data represent the value obtained from the fitting of mean data (3–4 experiments) as described in detail in the legend of Figure 1b.
ND: Not determined. [a]Jansen *et al.*, 1994.

Functional studies The electrically evoked tritium overflow was inhibited in a concentration-dependent manner by histamine and the H_3 agonist, (R)-α-methylhistamine (Table 1, Figure 1b). H_3-antagonists inhibited the (R)-α-methylhistamine effect with potencies comparable to their affinity for the [^{125}I]-iodophenpropit binding sites (Table 1). Clozapine (1 μM) shifted the (R)-α-methylhistamine concentration-response curve to the right (Figure 1b) yielding an apparent K_B value of 79.5 nM. Clozapine, itself, did not affect the stimulated (104 ± 1%; n = 3, mean ± s.e.mean) or the basal release of [^{3}H]-5-HT (100 ± 2%; n = 3, mean ± s.e.mean).

Discussion Clozapine, an atypical neuroleptic, has therapeutic efficacy in the treatment of drug-resistant schizophrenia (Baldessarini & Frankenburg, 1991). High affinity interactions of clozapine with 5-hydroxytryptamine 5-HT_2 and 5-HT_1 receptors, α_1- and α_2-adrenoceptors, histamine H_1, dopamine D_1 and D_4, and muscarinic receptors have been reported (Baldessarini & Frankenburg, 1991). Yet, in comparison to the typical neuroleptics, clozapine shows lower *in vitro* affinity for striatal dopamine D_2 receptors.

In the present work, we observed an intriguing interaction of clozapine with the histamine H_3 receptor, where clozapine acts as an H_3 antagonist. Other neuroleptics showed low affinity for the H_3 receptor.

The interaction of the non-imidazole compound, clozapine with the H_3 receptor is striking, since the imidazole moiety was thought to be required for high affinity H_3 binding.

In man, plasma concentrations of clozapine associated with clinical responses are approximately 0.6–1.2 μM (Baldessarini & Frankenburg, 1991). In rat, with doses higher than 5 mg kg^{-1} and within the therapeutic range in man, clozapine levels in the brain averaged 29 fold higher than corresponding serum drug levels (Balderassini *et al.*, 1993). Assuming total serum levels of 0.9 μM and 95% protein bound, total brain levels of 1.1 μM can be obtained. If the K_i value for clozapine at the rat H_3 receptor is similar for its human counterpart, brain clozapine concentrations might be high enough for a functional interaction with the H_3 receptor under clinical conditions. In conclusion: clozapine, showed a higher affinity for the histamine H_3 receptor than several other neuroleptics. Functionally, it revealed an antagonistic behaviour.

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Research report

Enhancement of prepulse inhibition of startle in mice by the H_3 receptor antagonists thioperamide and ciproxifan

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Abstract

Histamine H_3 receptor antagonists/inverse agonists have been proposed as potential therapeutic agents for the treatment of a number of neurological disorders ranging from attention deficit hyperactivity disorder and Alzheimer's disease to narcolepsy and schizophrenia. With respect to the latter, schizophrenic patients typically exhibit impaired prepulse inhibition (PPI) of startle, a reflex that can be modeled in many animal species. Certain strains of mice naturally display poor PPI and it was recently suggested that these mice might offer a new way to screen for novel antipsychotic compounds. To examine whether H_3 receptor antagonists might enhance PPI in mice with naturally occurring deficits, DBA/2 and C57BL/6 were tested in a startle paradigm with three prepulse intensities: 5, 10 and 15 dB above background. Both thioperamide and ciproxifan enhanced PPI in the DBA/2 strain; thioperamide also showed a trend towards enhancing PPI in C57BL/6. Risperidone, an atypical antipsychotic, enhanced PPI in both the DBA/2 and the C57BL/6 strain. These data confirm previous reports describing a natural deficit in PPI in some mouse strains that is amenable to enhancement with known antipsychotics. Further, these data suggest that H_3 receptor antagonists/inverse agonists have anti-psychotic potential for disorders such as schizophrenia.

Keywords: Prepulse inhibition; Thioperamide; Ciproxifan; DBA/2; C57BL/6; H_3 receptor antagonist/inverse agonist; Schizophrenia

1. Introduction

In recent years the therapeutic potential of histamine H_3 receptor ligands for treating disorders of the central nervous system has received increased attention [2,15,22,29,39]. First identified in 1983 as a presynaptic autoreceptor for the neurotransmitter histamine [1], a growing body of literature also supports a role for H_3 receptors in regulating the release of a number of additional neurotransmitters such as acetylcholine, dopamine, glutamate, noradrenaline, and serotonin [2,11]. Consistent with a localization within brain regions including the hippocampus, cerebellum, and cortex, in vitro and in vivo studies suggest that H_3 receptor ligands may have potential therapeutic use for treating attention deficit hyperactivity disorder, Alzheimer's disease and other neurological disorders with deficits in cognitive function [2,22,23,29,31].

Schizophrenia is a debilitating disorder often characterized by structural and functional abnormalities in brain regions involved in cognitive, emotional and motivational aspects of behavior (for a review, see [21]). While it is difficult to model all of the deficits observed in schizophrenia in a single animal model, much work has been devoted to developing models of specific dysfunctions. For example, deficits in attention and cognition observed in schizophrenics may be linked to impairments in sensorimotor gating ([25]; for a review, see [19]). Reductions in sensorimotor gating are also found in patients with Tourette's syndrome [6] and Huntington's disease [36]. One way to assess deficits

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in sensorimotor gating is to measure prepulse inhibition (PPI) of the startle response, a cross-modal phenomenon in which a startle response (in most cases to an acoustic noise) is inhibited when preceded by a weaker stimulus. Reduced levels of PPI compared to normal controls are believed to reflect the deficient sensorimotor gating that underlies sensory flooding and cognitive fragmentation in schizophrenic patients [19,25].

Prepulse inhibition can be modeled in a variety of additional species including mice, rats, cats and monkeys [14]. Traditionally, deficits in PPI in animal models have been induced using pharmacological manipulations including dopamine and serotonin receptor agonists, and non-competitive NMDA receptor antagonists; attenuation of these drug-induced impairments is hypothesized to reflect antipsychotic-like potential. There are some interpretive caveats to such models, however, and recently it was suggested that evaluation of compounds in mouse strains exhibiting a naturally occurring deficit in PPI may represent a more attractive alternative for testing putative antipsychotics with novel mechanism(s) of action [24,28,30]. Indeed, several clinically effective antipsychotics such as haloperidol [24,30], risperidone, and clozapine [30] have proved efficacious in this regard.

Central histaminergic systems have been implicated in the pathophysiology of schizophrenia [13], although it is not clear what receptor subtype might be mediating this relationship. Given that H_3 receptor antagonists/inverse agonists consistently improve cognition in animal models [14,15], and given that H_3 receptors are localized to brain regions implicated as having a functional role in these behaviors, we investigated the potential of the H_3 receptor antagonists/inverse agonists thioperamide and ciproxifan to enhance PPI of the startle response in DBA/2 and C57BL/6 mice that are known to exhibit naturally occurring deficits in PPI when compared to other strains [24,28,30].

2. Material and methods

2.1. Animals

C57BL/6 and DBA/2 mice (5–7 weeks old) were obtained from Charles Rivers Laboratories (USA). Animals were group housed up to 10 per large colony cage (52 cm × 28 cm × 20 cm) under a 12-h light:12-h dark schedule (lights on at 06:00 h), with food and water available ad libitum. Animals were acclimated to our animal facilities for a period of one week before commencement of experimental procedures. Animals were tested in the light phase. All experiments were conducted in accordance with Abbott Animal Care and Use Committee and National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2. Experimental equipment

DBA/2 and C57BL/6 mice were tested in either San Diego Instruments equipment (SRLAB, San Diego Instruments, San Diego, CA, USA), or Hamilton Kinder equipment (SM100 version 4.1, Poway, CA, USA). San Diego Instruments chambers (eight chambers total) consisted of a Plexiglas cylinder (5 cm diameter × 12.8 cm length) mounted on a platform in a ventilated, sound-attenuated cubicle. A piezoelectric device attached to the underside of the platform holding the cylinder transduced startle responses into analog signals. The signals were then digitized and stored by a computer controlling the timing of the various stimuli and the recording of data. A loudspeaker located in each chamber delivered a constant background noise (65 dB), and the acoustic stimuli. Testing in the Hamilton Kinder equipment was conducted in eight startle chambers, with each chamber containing a Plexiglas rectangle with an adjustable ceiling housed in a ventilated, sound-attenuated cubicle. The ceiling was adjusted on an individual basis to allow for adequate headroom but no rearing or extensive locomotion. The chamber was placed over an anchor plate attached to a piezoelectric disk to transduce startle responses for storage and analysis by computer. A loudspeaker located in each chamber delivered the background noise (65 dB), and the acoustic stimuli. A constant white noise was maintained in the experimental room for the duration of each experiment in either set of equipment by a white noise generator (Radio Shack, USA and Lafayette Instrument Co., USA).

2.3. Experimental procedures

Mice were individually placed in the startle chamber with a background white noise of 65 dB and left undisturbed for 5 min (total session duration was approximately 22 min). Following the 5-min acclimation period four successive 120 dB, 40 ms trials were presented. These trials were not included in analyses. Mice were then presented with five different trial types: startle pulse (120 dB, for 40 ms), no stimulus, or prepulse stimulus of one of three sound levels (70, 75, or 80 dB) for 20 ms, followed 100 ms later by an acoustic startle (120 dB for 40 ms). A total of 12 trials under each condition were delivered in a random sequence and all trials were separated by a variable inter-trial interval of 5–25 s. Finally, this sequence ended with the presentation of four 120 dB, 40 ms trials (not included in the analysis). Three prepulse levels were selected based on the observation that this procedure maximized the ability to detect changes in PPI.

In the startle alone trials, the basic auditory startle (startle response) was measured, excluding the first and last blocks of four pulse alone trials presented. In the prepulse plus startle trials, PPI was calculated as a percentage score for each acoustic prepulse trial type using the formula: $100 - [((\text{startle response for prepulse} + \text{pulse})/(\text{startle response for pulse-alone})) \times 100]$. For all analyses the max-

imum amplitude was used. Data from the no-stimulus trials are not included in the results section, as the values were negligible relative to values on trials containing startle stimuli.

2.4. Drugs

Independent groups of mice (each mouse was tested only once) received risperidone (Sigma, USA), ciproxifan (synthesized at Abbott Laboratories, Chicago, USA) or thioperamide (Tocris Cookson Inc., USA). Risperidone (0.3 or 1.0 mg/kg) was dissolved in acetic acid, brought to a pH of 5.0, and then brought to a final volume in sterile water (pH 5 to 6). Thioperamide was dissolved in saline (0.9% w/v, Abbott Laboratories, USA), and doses of 1, 3, 10, 15 or 30 mg/kg were administered. Ciproxifan (0.3, 1, 3 or 10 mg/kg) was dissolved in 0.1N HCl, brought to pH 5.0, and then brought to final volume in saline (pH 5 to 6). Compounds were administered intraperitoneally (i.p.) in an injection volume of 10 ml/kg, with a 30-min pretreatment time.

2.5. Statistical analysis

Data for each drug in each strain were analyzed using a one-way analysis of variance (ANOVA) with treatment as the factor. Significant effects as indicated by the ANOVA were investigated using Dunnett's post hoc analyses. Criteria for significance was set at $P < 0.05$.

3. Results

3.1. Effects of risperidone on PPI in DBA/2 and C57BL/6 mice

To assess the predictive validity of this model, we administered doses of 0.3 and 1 mg/kg (selected from [30]) of the antipsychotic risperidone to mice from both strains. These results are presented in Fig. 1.

There was a significant response to risperidone in DBA/2 mice (Fig. 1A) as indicated by a significant ANOVA ($F(2, 26) = 8.336$, $P < 0.01$), with Dunnett's post hoc analyses indicating a significant potentiation in PPI following the administration of 0.3 and 1.0 mg/kg risperidone. Panel 1B depicts the response across prepulse intensities.

Effects of 0.3 and 1.0 mg/kg of risperidone on PPI in C57BL/6 mice are presented in Fig. 1C. An ANOVA on these data indicated a significant effect of treatment ($F(2, 27) = 8.20$, $P < 0.01$). Dunnett's post hoc analyses indicate a significant potentiation following the administration of 1.0 mg/kg risperidone. Panel D illustrates the response of C57BL/6 mice across prepulse intensities.

There were significant effects on startle response to the 120 dB pulse alone following the administration of risperidone in DBA/2 (Table 1; $F(2, 26) = 5.58$, $P < 0.01$) and C57BL/6 mice (Table 2; $F(2, 27) = 9.89$, $P < 0.01$). Post hoc analysis identified a significantly reduced startle alone

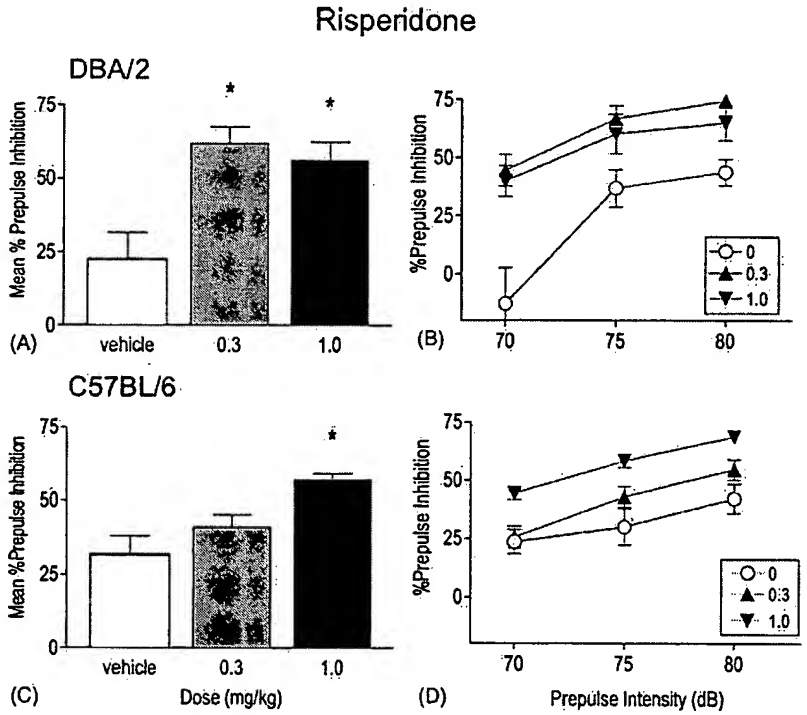

Fig. 1. Effect of risperidone (0.3 and 1.0 mg/kg; $N = 9$–10 per dose) on PPI in DBA/2 and C57BL/6 male mice. (A) Mean ± S.E.M. percent PPI in DBA/2 mice. DBA/2 mice receiving 0.3 and 1.0 mg/kg risperidone showed a significant enhancement relative to vehicle treated controls; (B) percent PPI after three prepulse intensity levels (70, 75 and 80 dB) in DBA/2 mice; (C) mean ± S.E.M. percent PPI in C57BL/6 mice; treatment with 1.0 mg/kg in this strain produced a significant enhancement; (D) response to risperidone in C57BL/6 mice across prepulse intensities. (*) indicates significance from the corresponding vehicle treatment (Dunnett's test after significant ANOVA).

Table 1
Response (mean ± S.E.M. startle amplitude to the twelve 120 dB alone trials) to risperidone (0.3 or 1 mg/kg), thioperamide (10 or 30 mg/kg) and ciproxifan (doses: 1, 3 or 10 mg/kg) in DBA/2 mice

Mean Startle Response in DBA/2 mice	
Risperidone (mg/kg)	
0	0.344 ± 0.073
0.3	0.404 ± 0.094
1.0	0.080 ± 0.017*
Thioperamide (mg/kg)	
0	213.89 ± 69.13
10	269.30 ± 57.20
30	221.25 ± 69.13
Ciproxifan (mg/kg)	
0	186.25 ± 49.52
1	117.56 ± 23.43
3	194.90 ± 23.12
10	233.30 ± 19.79

* $P < 0.05$ compared to vehicle control (Dunnett's test). The risperidone study was conducted in the Hamilton-Kinder equipment, so the recorded values are Newtons; for the other experiments the units are arbitrary.

response in DBA/2 mice treated with 1.0 mg/kg when compared with vehicle-treated control mice, and a significant decrease in startle response in the C57BL/6 mice following the administration of 0.3 and 1.0 mg/kg risperidone.

3.2. Effects of thioperamide and ciproxifan on PPI in DBA/2 and C57BL/6

When data were analyzed from DBA/2 mice treated with thioperamide (Fig. 2A), statistical analysis indicated a significant main effect of dose ($F(2, 26) = 6.97$, $P < 0.01$), with Dunnett's comparisons indicating a significant enhancement following the administration of 30 mg/kg thioperamide. Fig. 2B illustrates the response across prepulse intensities.

Table 2
Response in C57BL/6 mice (mean ± S.E.M. startle amplitude) to risperidone (0.3 or 1 mg/kg), thioperamide (10 or 30 mg/kg) or ciproxifan (doses: 1, 3 or 10 mg/kg) on the mean startle amplitude (response to the average of the 12, 120 dB trials)

Mean startle response in C57BL/6 mice	
Risperidone (mg/kg)	
0	1.47 ± 0.206
0.3	0.943 ± 0.138*
1.0	0.498 ± 0.106*
Thioperamide (mg/kg)	
0	1.855 ± 0.192
10	1.475 ± 0.115
30	1.483 ± 0.093
Ciproxifan (mg/kg)	
0	1.757 ± 0.147
1	1.132 ± 0.203
3	1.253 ± 0.185
10	1.133 ± 0.176*

The recorded values are Newtons. * $P < 0.05$ compared to vehicle control (Dunnett's test).

Fig. 2 also shows the magnitude of PPI in C57BL/6 mice following the administration of thioperamide (Fig. 2C; 10 and 30 mg/kg). An ANOVA revealed a trend towards a significant effect of treatment ($F(2, 27) = 2.88$, $P = 0.07$). Fig. 2D depicts the response to thioperamide in C57BL/6 mice across prepulse intensities, demonstrating that the nature of the trend is most likely due to the increasing response to thioperamide at the higher dB intensities.

Ciproxifan also enhanced PPI in DBA/2 mice (Fig. 3A). The ANOVA revealed a significant effect ($F(3, 33) = 3.58$, $P < 0.05$), with post hoc analyses indicating both 1 and 10 mg/kg reached significance relative to the values of vehicle controls. Further analysis of the response across prepulse intensities (Fig. 3B) illustrates that the response of 3 mg/kg was similar to that observed following the administration of 1 and 10 mg/kg, although it did not reach significance.

There was no significant effect of 1, 3 and 10 mg/kg ciproxifan on prepulse inhibition in C57BL/6 mice (Fig. 3C; ($F(3, 35) = 0.759$, $P = 0.53$). Fig. 3D illustrates the response to ciproxifan across prepulse intensities.

There were no significant effects on the startle response to the 120 dB pulse alone in DBA/2 mice (Table 1) following administration of thioperamide ($F(2, 26) = 0.26$, $P = 0.78$) or ciproxifan ($F(3, 33) = 1.60$, $P = 0.21$). Similarly, there were no significant effects of thioperamide on the startle response to a 120 dB pulse alone in C57BL/6 mice (Table 2; $F(2, 27) = 2.4$, $P = 0.11$), although there was a decrease in startle response in mice administered ciproxifan ($F(3, 35) = 2.84$, $P = 0.05$), with Dunnett's post hoc analyses indicating mice receiving 10 mg/kg demonstrating significantly reduced startle response.

4. Discussion

Schizophrenia is a complex neurological disorder with multiple facets. Consequently, there is no one definitive animal model of the various social, motor and cognitive deficits characteristic of schizophrenic patients. Specific aspects of this neurodevelopmental disorder, however, are modeled readily in animals. One feature common to schizophrenics [4,5] as well as patients afflicted with obsessive-compulsive disorder, Tourette's syndrome [6] and Huntington's disease [36], is reduced sensorimotor gating, a neural mechanism that inhibits extraneous sensory, cognitive, and motor information to allow proper integration of appropriate input. Such gating can be operationally assessed a number of different ways in various species ranging from mice and rats to monkeys and humans [8,9,14]. One common method is by making use of the startle reflex, a stereotyped motor 'flinching' response to a sudden, intense acoustic stimulus. Presentation of lower intensity acoustic stimuli immediately prior to a high intensity acoustic startle stimulus attenuates the organism's response to the latter, a phenomenon referred to as prepulse inhibition of startle or PPI, which is

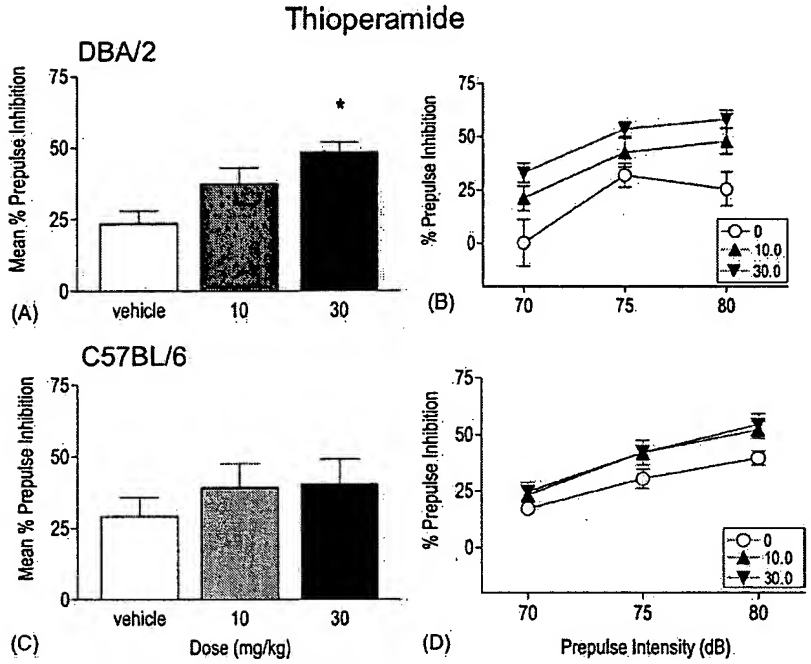

Fig. 2. Effect of thioperamide (10 and 30 mg/kg; $N = 9$–10 per dose) on PPI in DBA/2 and C57BL/6 male mice. (A) Mean ± S.E.M. percent PPI in DBA/2 mice; (B) response to thioperamide in DBA/2 mice across prepulse intensities; (C) mean ± S.E.M. percent PPI in C57BL/6 mice; (D) percent PPI after three prepulse intensity levels (70, 75 and 80 dB) in C57BL/6 mice. (*) indicates significance from the corresponding vehicle treatment (Dunnett's test after significant ANOVA).

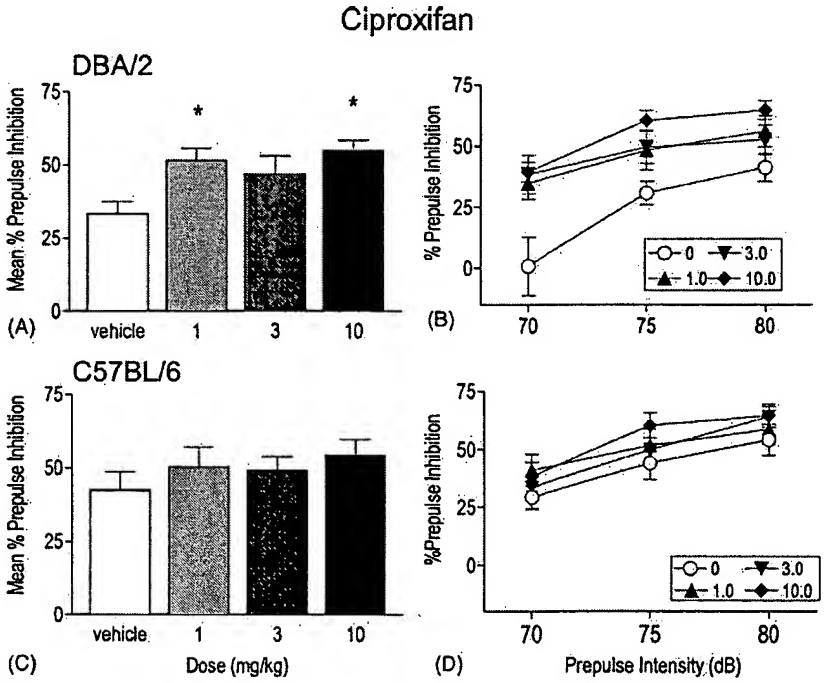

Fig. 3. Effects of ciproxifan (1, 3, and 10 mg/kg; $N = 8$–10 per dose) on PPI in DBA/2 and C57BL/6 male mice. (A) Mean ± S.E.M. percent PPI in DBA/2 mice; (B) PPI after three prepulse intensity levels (70, 75 and 80 dB); (C) mean ± S.E.M. percent PPI in C57BL/6; (D) PPI across prepulse intensities. (*) indicates $P < 0.5$ from the corresponding vehicle treatment (Dunnett's test).

impaired when evaluated clinically in schizophrenics [3]. Assessment of PPI in rodents has been proposed as a model of this feature of schizophrenia [18,35].

High densities of H_3 receptors are found in the cortex, striatum and hippocampus of both the rat [12] and the human [38] brain, lending support to a functional role for these receptors in sensory and cognition processing. A number of previous studies using a range of H_3 receptor antagonists/inverse agonists, including thioperamide and ciproxifan, have demonstrated positive effects in animal models of attention and short-term memory such as repeated acquisition, inhibitory avoidance [15], five-choice, serial reaction time task [22] and social recognition [16,33]. To date and to the best of our knowledge, however, no positive effects have been demonstrated for H_3 receptor antagonists/inverse agonists in animal models in which deficits in sensorimotor gating are apparent.

We therefore investigated whether thioperamide and ciproxifan could positively influence PPI in two different mouse strains. Mice were chosen over rats since recent evidence suggests that, provided the proper conditions are selected, known antipsychotics can directly enhance PPI in mouse strains that normally exhibit poor PPI responding [24,28,30]. We reasoned that these conditions might be more analogous to the impaired responding observed in untreated schizophrenics. Consistent with our expectations, among the strains tested, DBA/2 mice exhibited the poorest PPI responding. Reasoning that this may provide an optimal window in which to observe pharmacological enhancement, we evaluated both thioperamide and ciproxifan in this strain and observed the greatest response to the potentiating effects of H_3 antagonists/inverse agonists relative to C57BL/6 mice. Significant enhancements of PPI responding were observed for thioperamide (Fig. 2), ciproxifan (Fig. 3), and risperidone (Fig. 1).

Drug-treated C57BL/6 mice showed a trend towards enhancement in PPI following administration of thioperamide, but did not show a significant response to ciproxifan. A significant enhancement of the PPI of startle response was also observed in C57BL/6 mice following administration of the antipsychotic risperidone (Fig. 1; doses selected from [30]). These data are in agreement with other reports that antipsychotics could facilitate PPI in this strain [24,30], but contrast work by Oliver and coworkers (2001) who report no significant increase in PPI in C57BL/6J mice receiving similar doses of risperidone. Further, imipramine (an antidepressant) did not significantly enhance PPI in the C57BL/6 mice (unpublished data), supporting findings of Ouagazzal and coworkers (2001) that antidepressants were not effective at potentiating PPI in this strain.

Although two different sets of equipment were used in these experiments, this does not seem sufficient to explain the observed differences in drug responsiveness. Risperidone was conducted in the same apparatus for both strains, and the response of the DBA/2 mice was greater than that observed with the C57BL/6, consistent with observations following the administration of H3 antagonists. It is important to note that while the design and approach to testing startle and prepulse inhibition of the startle response is different between the two sets of equipment, fundamentals such as the transducer is the same between the two apparatus (personal correspondence with Mike Kinder). Thus, there is little reason to suggest that these results are equipment dependent.

With risperidone, enhancement of the PPI of startle response in DBA/2 mice was only seen with doses that significantly reduced the startle response. This raises a general concern that improvement of PPI responding might be confounded by drug-induced reductions of the basal startle response. To address this issue, there are numerous studies demonstrating the independence of drug effects on PPI and basal startle in both mice and rats [10,17,32,35,37]. Further, it has been suggested that the acoustic startle reflex, and its inhibition by lower intensity prepulses, involve different genetically defined physiological processes [24,32]. Paylor and Crawley (1997) found no correlation, across a panel of 13 inbred strains, between the level of PPI and the amplitude of the startle response. Further, our current results with H_3 receptor antagonists/inverse agonists also support this dissociation between drug effects on startle response and PPI. For example, DBA/2 mice pretreated with 10 mg/kg ciproxifan showed no significant differences in startle response, but did show a significant enhancement of PPI. Following treatment with thioperamide, again DBA/2 mice did not show a significant change in basal startle response relative to vehicle treated mice, but did show a significant enhancement of PPI.

H_3 receptor antagonists/inverse agonists have demonstrated positive effects in several rodent cognition tasks [2,15,22,33], so it is conceivable that such compounds might be effective in treating the multiple cognitive and gating deficits observed in schizophrenic patients. The ability of putative antipsychotics to attenuate the hyperactivity induced by dopaminergic agents is another model frequently used to assess potential for therapeutic utility in schizophrenia. It was recently reported that rodents treated with methamphetamine [20,26] show a hyperactivity of dopaminergic transmission that is accompanied with an enhanced activity of histaminergic neurons. Further, both thioperamide and ciproxifan attenuated the locomotor activation induced by dopaminergic agonists [7,26], supporting the current results.

Additional evidence supports a role for the histaminergic system in schizophrenia. For example, overdose of first generation H_1 receptor antagonists produced toxic psychoses with hallucinations resembling those seen in schizophrenia [34]. One concern with neuroleptics is their side effects, especially the catalepsy seen with the typical antipsychotics such as haloperidol. The studies with H_1 receptor antagonist [34] suggest that investigating liabilities associated with histaminergic approaches may be important. Pillot and coworkers (2002) recently demonstrated that co-administration of ciproxifan and the typical antipsychotic haloperidol potentiated haloperidol-induced locomotor hypoactivity and catalepsy. However, there are some

important caveats to these data: first, it is not clear whether the locomotor hypoactivity and catalepsy potentiating effects of ciproxifan apply to other antipsychotics and second, it is also not clear whether this is a phenomenon restricted to ciproxifan. Atypical antipsychotics stimulate histamine neurons, an effect that may underlie the pro-cognitive properties of atypical antipsychotics [27]. Thus, the ability of H_3 receptor antagonists/inverse agonists to assist in treating sensorimotor gating deficits seen in schizophrenia from potentiating side effects are most likely dissociable, but should be investigated carefully.

In summary, the present study yielded three main results. First, we have confirmed that DBA/2 mice may be the most sensitive mouse strain to use in investigating the potential of compounds at novel targets; the strain previously shown to exhibit the greatest natural deficit of the C57BL/6 and DBA/2 strains [28]. Second, we expand upon these data by demonstrating for the first time enhancement of PPI with two different H_3 receptor antagonists/inverse agonists in a model of sensorimotor deficits frequently observed in schizophrenics, with both ciproxifan and thioperamide producing the most robust enhancement of PPI in DBA/2 mice. Lastly, we have demonstrated that risperidone was also effective in enhancing PPI in both strains, providing support for this model as a viable tool for screening putative novel antipsychotics. Our current findings thus provide the first direct evidence that blockade of the H_3 receptor with antagonists/inverse agonists may offer a new approach to the treatment of sensorimotor gating deficits observed in schizophrenic patients.

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NEURONAL HISTAMINE DEFICIT IN ALZHEIMER'S DISEASE

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Abstract—Histamine is known to be a neurotransmitter in the brain, but it has not been clearly implicated in major diseases. All histaminergic neurons reside in the posterior hypothalamus and innervate most brain areas, which is compatible with the concept that histamine is involved in general central regulatory mechanisms. A sensitive high-performance liquid chromatographic fluorimetric method was used to measure histamine contents in *post mortem* Alzheimer brains and age-matched controls. The cellular storage sites and distribution of histaminergic nerve fibers were examined with a specific immunohistochemical method. The histamine content was significantly reduced in the hypothalamus (42% of control value), hippocampus (43%) and temporal cortex (53%) of Alzheimer brains. Differences in other cortical areas, putamen and substantia nigra were not significant. Histamine-containing nerve fibers were found in the hippocampus, parahippocampal gyrus and subiculum of both Alzheimer brains and controls. No histamine-containing mast cells were seen in these temporal structures.

Histamine in the human temporal lobe is stored in nerve fibers originating from the posterior hypothalamus, and not in mast cells. Decrease in brain histamine may contribute to the cognitive decline in Alzheimer's disease directly or through the cholinergic system. Development of drugs that penetrate the blood–brain barrier and increase histaminergic activity might be beneficial in Alzheimer's disease. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: hippocampus, tuberomammillary nucleus, hypothalamus, carbodiimide.

Recent studies have shown that histamine may regulate higher brain functions by at least two mechanisms. It enhances hippocampal *N*-methyl-D-aspartate-mediated synaptic currents in cultured hippocampal neurons,[2,26] an effect apparently mediated through the polyamine-binding site on the *N*-methyl-D-aspartate receptor complex,[26] and switches thalamic neuronal activity from rhythmic burst firing to single-spike activity through histamine H_1 and H_2 receptors, thus promoting accurate transmission of thalamocortical relay neurons and processing of sensory inputs and cognition.[12] Thus, histamine mediates its effects not only through specific histamine H_1, H_2 and H_3 receptors (for recent reviews see Refs 9 and 23), but also through modulatory actions on other receptors.

Histamine is widely distributed in the human brain.[10] The cell bodies of histaminergic neurons lie in the posterior hypothalamus, where about 64,000 cells form a loose group in the nucleus tuberomamillaris.[1] These neurons have not been found in other areas of the human brain.[17] The general organization of the histaminergic system is similar in all vertebrates studied so far: cell bodies are found in the tuberomammillary nucleus,[18] which provides almost all parts of the CNS with varicose fibers containing histamine. In the human CNS, histamine-containing projections extend at least to different parts of the cerebral cortex[17] and cerebellar cortex.[19] Other areas of the human brain have not been studied. In Alzheimer's disease, neurofibrillary tangles were found to colocalize with histamine in the tuberomammillary areas in the posterior hypothalamus,[1] and significantly reduced neuron numbers in the tuberomammillary nucleus have been reported in Alzheimer's brains.[15]

Previous studies on histamine in Alzheimer's disease report conflicting results. In one study, significant increases in histamine concentrations were found in almost all brain areas except for the corpus callosum and globus pallidus,[4] whereas another study reported significant decreases in the frontal, temporal and occipital cortices and in the caudate nucleus.[11] The purpose of this study was to analyse the possible changes in the brain histaminergic system using a very sensitive high-pressure liquid chromatographical method and patient material in which the *post mortem* storage times and temperatures are well controlled. As the organization of the human histaminergic system in the temporal lobe has not been analysed, the cellular storage sites of histamine in normal and Alzheimer brains were also revealed

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Abbreviations: PBS, phosphate-buffered saline.

Table 1. Characteristics of patients analysed for histamine content

	Alzheimer's disease	Controls
Age (years)	81.6 ± 9.5 (61–95)	82.2 ± 3.9 (76–88)
Sex (F/M)	7/2	4/1
Post mortem time (h)	17.4 ± 7.2 (6–29)	17.6 ± 7.2 (8–30)

Data are expressed as mean ± S.D.; ranges are given in parentheses.

using immunocytochemistry. This is important, because brain histamine is stored in both neurons and mast cells.[18,20,23]

EXPERIMENTAL PROCEDURES

Samples for histamine measurements were collected from autopsies in the Turku Central University Hospital. A permit for the study was obtained from the National Board of Medicolegal Affairs. The brains were dissected, weighed, frozen on dry ice and kept at −70°C. The patient data are shown in Table 1. For analysis, the samples were analysed using high-performance liquid chromatography fluorometry, as described earlier.[25] On neuropathological examination, the Alzheimer patients showed numerous senile plaques and tangles in the neocortex and hippocampus. All patients fulfilled the requirements for definite Alzheimer's disease according to the CERAD criteria.[13]

For immunohistochemistry, autopsy samples from fresh brains were obtained. Five normal male brains were analysed (age 64.4 ± 14 years, range 49–78, *post mortem* time 44 ± 24 h; cause of death: cardiac infarct). The two Alzheimer brains were also from males (ages 69 and 67 years, *post mortem* times 10.5 and 29 h; cause of death: pneumonia). The brains were dissected into 1-cm-thick slices and fixed by immersion in 4% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma, St Louis, MO, U.S.A.) and 1% paraformaldehyde in 0.1 M sodium phosphate buffer. After extensive washes in phosphate-buffered saline (PBS), 40-μm-thick free-floating cryostat sections were first treated with 1% hydrogen peroxide in 0.01 M PBS (pH 7.4) for 30 min to remove endogenous peroxidase. To improve the immunostaining the paraformaldehyde was removed by treating the sections with 0.5% sodium borohydride in PBS for 30 min. Sections were then incubated with specific histamine antiserum[17,20] diluted 1:10,000 in PBS containing 0.25% Triton X-100 for 48 h, then washed 2 × 10 min in PBS. Immunoreactivity was visualized with the biotin–streptavidin method (Vectastain Elite Kit, Vector, Burlingame, CA, U.S.A.) according to the manufacturer's instructions. The peroxidase reaction was visualized by incubating the sections in 0.025% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer for 30 min. Control sections were treated similarly, except that they were incubated with histamine antiserum preadsorbed with a histamine–ovalbumin conjugate (1–10 μg/ml). No immunoreaction was seen in these samples.

RESULTS

Histamine concentrations

To control the possible effect of *post mortem* time on the histamine content, samples of temporal cortex and hippocampus taken from normal brain on the first *post mortem* day were kept at 4°C, and the histamine content was measured from aliquots taken from these samples each day. A significant, nearly linear but highly variable (70% to 10-fold during six days in different samples) increase occurred after the first day, which called for careful control of *post mortem* times and conditions.

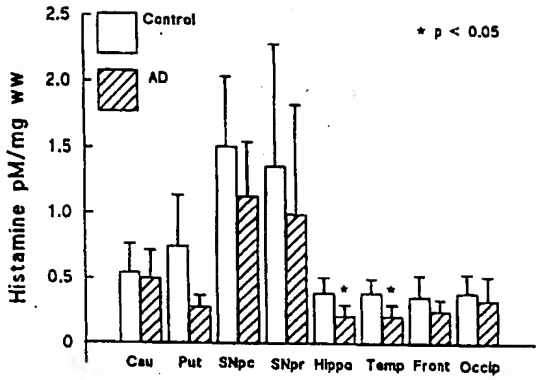

Fig. 1. Histamine concentrations in different brain areas of control brains (n=5) and Alzheimer's disease brains (n=9). Cau, nucleus caudatus; Put, putamen; Hippo, hippocampus; Temp, temporal cortex; SN_{PC}, substantia nigra, pars compacta; SN_{PR}, substantia nigra, pars reticulata; Front, frontal cortex; Occip, occipital cortex.

Table 2. Histamine concentration in the hypothalamus

Alzheimer's disease (n=9)	Controls (n=5)
2.13 ± 1.55	4.97 ± 2.30*

Values given are mean ± S.D. *P<0.05.

Histamine concentrations were significantly lower in the hypothalamus, hippocampus and temporal cortex of Alzheimer brains than in control brains (Fig. 1, Table 2). The differences in the prefrontal and occipital cortices, putamen, pars compacta and pars reticulata of the substantia nigra were not significant, although a tendency to reduced levels was seen. No difference in the concentrations was seen in the nucleus caudatus (Fig. 1).

Distribution of histamine-immunoreactive nerve fibers

All areas of the temporal lobe contained histamine-immunoreactive nerve fibers in normal brains. There was no obvious difference in the morphology or distribution of nerve fibers between normal brains and the two cases of Alzheimer's disease (Fig. 3).

Long varicose fibers immunoreactive for histamine entered the hippocampus via both fimbriae and the perforant pathway (Figs 2, 3). The density of these fibers in the fimbriae and subiculum was higher than in other areas of the hippocampus. Scattered fibers were seen in hippocampal fields CA1–CA3 and in the gyrus dentatus. Moderately dense fibers innervated all layers of the parahippocampal gyrus (Fig. 3e) and entorhinal cortex. No histamine-immunoreactive mast cells or capillary endothelial cells were seen in these brain areas.

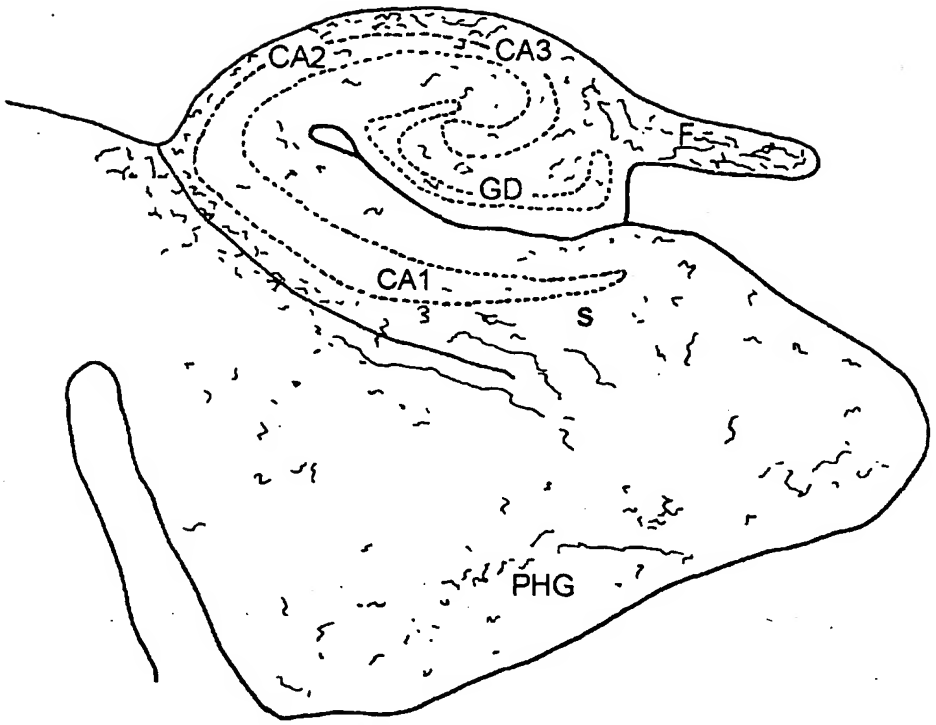

Fig. 2. Schematic distribution of histamine-immunoreactive nerve fibers in normal human hippocampal area. F, fimbriae; CA1–CA3, cornu ammonis, area 1–3; GD, gyrus dentatus; S, subiculum; PHG, parahippocampal gyrus.

DISCUSSION

Based on our findings, nerve fibers are the primary storage site of histamine in the human hippocampus and associated temporal lobe structures. It appears that the hypothalamic histaminergic neurons project to the hippocampus both through the subiculum and fimbriae, which is in agreement with previous findings in the rat and with abundant fiber bundles originating from the hypothalamic histamine-containing neurons in human brain.[1] The results of this study show a clear decrease in histamine concentrations in the hippocampus and temporal cortex in Alzheimer's disease. A similar significant difference was seen in the hypothalamus, where all histaminergic fibers originate.[1,17] Immunohistochemistry was used to reveal the neuronal location of histamine in these structures. Previous studies have shown accumulation of neurofibrillary tangles in the tuberomammillary nucleus and a decrease in neuron numbers in the same area in Alzheimer's disease.[1,15]

The decline in histamine levels observed in this study is in agreement with one previous study,[11] and differs from that of another study.[4] Lack of histamine-immunoreactive mast cells suggests that the observed changes occur in the neuronal pool. Obviously, the *post mortem* time and storage conditions are essential factors that contribute to previous conflicting results, as a significant increase in brain histamine concentration occurs with increasing *post mortem* time. *Post mortem* times need to be carefully controlled.

Although the cholinergic system is commonly considered to be the one most severely affected in Alzheimer's disease, loss of dopamine, serotonin and noradrenaline have also been reported in a number of studies (for review see, e.g., Ref. 6). It is interesting to note that tetrahydroaminoacridine, a non-specific cholinesterase inhibitor that affects cholinergic functions[16] and is found to be beneficial in Alzheimer's disease,[24] also increases the action potential duration of histaminergic neurons,[22] and inhibits histamine *N*-methyltransferase[5] and may thus affect histaminergic transmission in the brain. Decreased histaminergic input may also affect cholinergic activation of cortical and hippocampal neurons, as histamine excites cholinergic nucleus basalis neurons[8] and stimulation of the tuberomammillary histaminergic neurons increases hippocampal acetylcholine release in rats, an effect inhibited by an H_1 receptor antagonist, pyrilamine.[14] The presence of a widespread histaminergic neuronal system in the temporal lobe as shown here and in other parts of the cerebral cortex[17] may also have implications in other disorders involving cortical functions. Improvement of negative symptoms in schizophrenic patients has been reported after adminstration of histamine H_2 receptor blockers,[7] and histamine metabolites in the cerebrospinal fluid of some schizophrenics are elevated.[21]

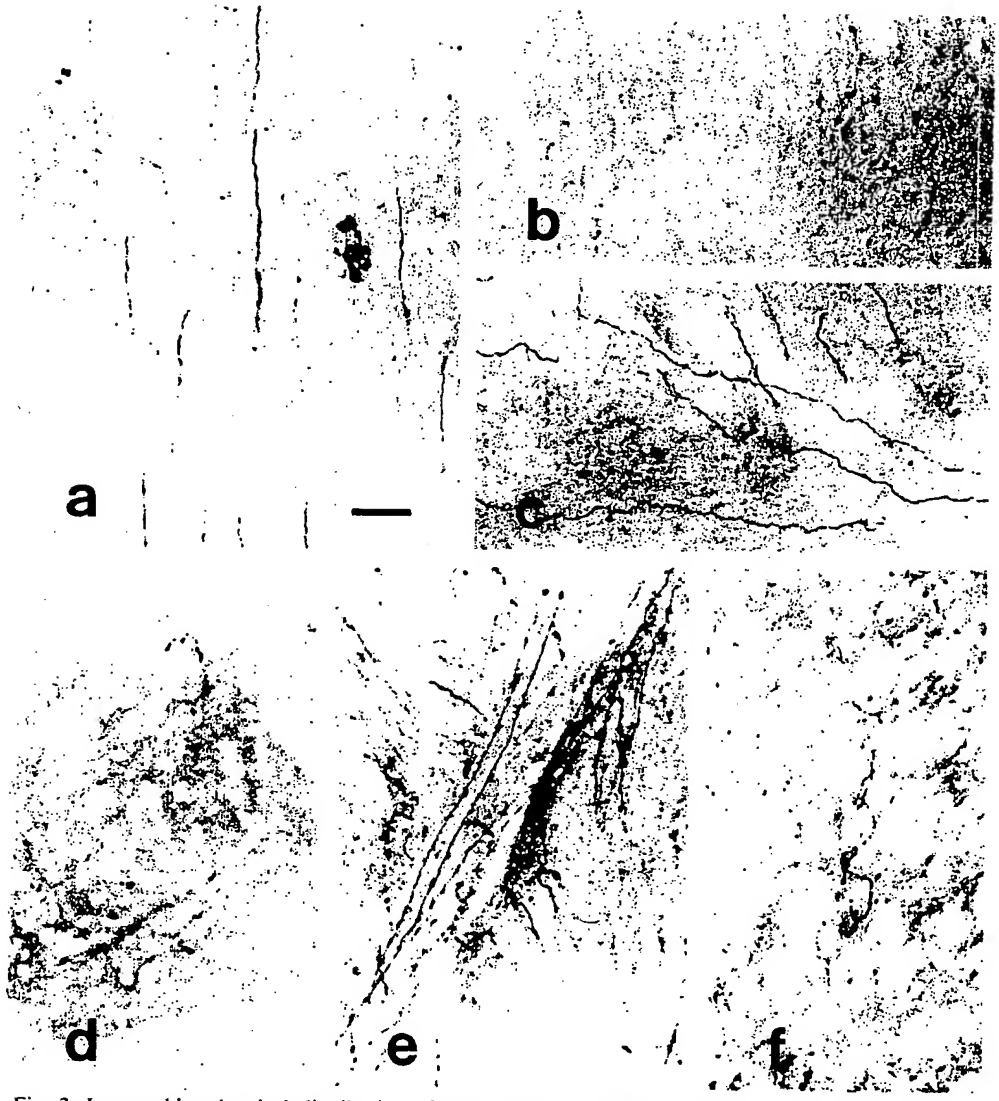

Fig. 3. Immunohistochemical distribution of histamine-immunoreactive fibers in human hippocampal area. Moderately dense fibers in the fimbriae (a), solitary thin varicose fibers in the pyramidal layer of the CA3 area (b) and long, varicose fibers in the subiculum (c) of an Alzheimer brain are shown. A single varicose fiber in the dentate gyrus (d), long varicose fibers in the alveus and subiculum (e), and terminal arborizations of histamine-immunoreactive fibers in the parahippocampal gyrus (f) in normal brain can be seen. Scale bar=50 μm.

CONCLUSION

Taken together, the results show that significant reductions, up to 55% in the hippocampus, are found in brain neuronal histamine content in Alzheimer's disease. It is suggested that lack of brain histamine may contribute to the cognitive decline in Alzheimer's disease. However, activation of different histamine receptors may exert different modulatory effects on other systems. For example, extended use of H_2 blocking agents has been reported to delay the onset of Alzheimer's disease among siblings at high risk.[3]

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Effects of Thioperamide, a Histamine H_3 Antagonist, on the Step-Through Passive Avoidance Response and Histidine Decarboxylase Activity in Senescence-Accelerated Mice

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Received 25 January 1994

MEGURO, K.-I., K. YANAI, N. SAKAI, E. SAKURAI, K. MAEYAMA, H. SASAKI AND T. WATANABE. *Effects of thioperamide, a histamine H_3 antagonist, on the step-through passive avoidance response and histidine decarboxylase activity in senescence-accelerated mice.* PHARMACOL BIOCHEM BEHAV 50(3) 321-325, 1995. – The effect of thioperamide, a histamine H_3 receptor antagonist, on learning and memory was studied in the senescence-accelerated mice-prone strain (SAM-P/8) and normal-rate aging strain (SAM-R/1). In a passive avoidance test, SAM-P/8 mice of 12 months showed significant impairment of learning and memory compared with SAM-R/1 mice of the same age. Thioperamide significantly improved the response latency in SAM-P/8 mice when injected intraperitoneally at a dose of 15 mg/kg. The histidine decarboxylase (HDC) activity in the forebrain was significantly lower in SAM-P/8 mice than in SAM-R/1 mice. Thioperamide administration significantly potentiated HDC activity in the forebrain of SAM-P/8 mice as well as improving learning and memory. These results suggest that central histaminergic neurons may be involved in learning and memory impairment of SAM-P/8 mice, although other possibilities are not ruled out.

Histamine Histamine H_3 receptor Thioperamide Brain histamine content Acquisition memory Passive avoidance Brain

IMMUNOHISTOCHEMICAL studies have demonstrated the existence of histaminergic neurons in the brain, which are concentrated in the tuberomammillary nucleus of the posterior hypothalamus, and which project efferent varicose fibers to almost all parts of the brain (13,23). Consistent with its wide-ranging output, the histaminergic neuron system regulates various activities of the brain, such as the arousal state, brain energy metabolism, locomotor activity, neuroendocrine, autonomic and vestibular functions, feeding, drinking, sexual behavior, and analgesia (20,24). Recent studies also revealed that endogenous histamine plays an important role in learning and memory (7).

The senescence-accelerated mouse (SAM), a murine model of accelerated aging, showed an early onset and irreversible advancement of senescence (12). There are two strains of SAM, SAM-P and SAM-R, respectively, which are prone and resistant to accelerated senescence. SAM-P/8, a substrain of SAM-P, shows marked age-accelerated decrease in life span, deterioration of the skin and hair, amyloid deposition, and a remarkable age-accelerated deterioration in learning passive avoidance, whereas a SAM-R/1, a substrain of SAM-R, does not exhibit these characteristics appreciably, showing a normal process of development and aging. Recently, we measured the level of histamine and activity of histidine decarboxylase

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(HDC, L-histidine carboxylyase, E.C.4.1.1.22) in SAM, and found that the central histaminergic neuron system was attenuated in SAM-P/8 mice, whereas the mast cell-derived histamine content of their brain increased with age (11).

Besides postsynaptic H_1 and H_2 receptors, presynaptic H_3 receptors have been proposed to control the synthesis and release of neuronal histamine (2,20,21). Thioperamide is a histamine H_3 receptor antagonist that increases the release of neuronal histamine and the activity of HDC in the brain. In this article we report the effects of thioperamide on learning and memory, measured by a passive avoidance test, and the HDC activity in SAM.

METHOD

Animals

SAM-P/8 and SAM-R/1 mice were bred under conventional conditions. The two strains of SAM used in this study were brought to our laboratory at about 2 months of age and kept at 24 ± 2°C, with commercial diet and tap water ad lib. Forty-six aged male SAM-P/8 mice (12–16 months) and 41 aged SAM-R/1 mice (12–17 months) were used in our passive avoidance experiments. Previous studies showed that age-related differences between the grading scores of senescence and behavioral performances of SAM-P/8 and SAM-R/1 mice were marked after 6 months of age. The SAM-P/8 mice used in our experiments showed some characteristic features of accelerated senescence. Young (4–6 weeks) and old (11–18 months) groups of both strains of mice were used in binding experiments.

Passive Avoidance

The step-through passive avoidance response was examined between 2100 and 2200 h every day. The apparatus consisted of two compartments; one was illuminated with 60 W (150 × 100 mm with a height of 100 mm to top of chamber) and the other was dark. The compartments were separated by a guillotine door (20 × 20 mm). When the mice were placed into the illuminated safe compartment, they tended to escape to the dark compartment through the door and stand on a grid floor. When all four paws were on the grid, a scrambled constant current (0.3 mA) and constant voltage (50 V, 50 Hz) foot shock was delivered to the grid for 3 s. The mice could escape from the shock only by stepping back into the safe illuminated compartment. Then the mice were returned to their home cages. Passive avoidance learning was repeated on the second, third, fourth, and fifth days in the same way as in the first trial, and the response latency for entering the dark compartment was measured (17). Thioperamide (7.5 or 15 mg/kg as free base) dissolved in 0.2 ml of saline was administered IP 60 min before each trial of passive avoidance. The latency of mice that did not move into the dark compartment during the observation period was calculated to be 300 s.

Determination of Brain HDC Activity

Mice were killed by decapitation 3 h after the treatment of thioperamide on the last day; their brains were quickly removed and divided on ice into three regions: the forebrain (including the cortex, hippocampus, and striatum) and midbrain (the thalamus, hypothalamus, and colliculi).

HDC activities of forebrains and midbrains were measured as described previously (11). Briefly, brains were homogenized in 10 vol. of HDC solution (100 mM potassium phosphate, pH 6.8, 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 1% polyethyleneglycol, and 100 μg/ml phenylmethanesulfonylfluoride) in a Polytron homogenizer (Kinematica, Lucern, Switzerland) at a maximum setting for 20 s on ice. The homogenate was centrifuged at 10,000 × *g* for 30 min, and the supernatant was dialyzed against the HDC solution overnight. The HDC reaction was started by adding 0.5 mM L-histidine. The histamine produced during incubation (3 h) at 37°C was measured by the HPLC fluorescence method. Histamine was separated by a cation-exchange column, labeled with *o*-phthalaldehyde, and detected fluorometrically with a fluorometer (Tosoh, Tokyo, FS-8010) using excitation and emission wavelengths of 360 and 450 nm, respectively.

[^{3}H](R)α-Methylhistamine Binding to Histamine H_3 Receptors in Brains of SAM-P/8 and -R/1 Mice

[^{3}H](*R*)α-Methylhistamine binding was measured by the method described by Arrang et al. (2). Forebrains of SAM-P/8 and -R/1 mice were homogenized in 40 vol. of 50 mM Na^+-K^+ phosphate buffer, pH 7.5, and the homogenate was centrifuged at 50,000 × *g* for 20 min. The precipitate was resuspended in fresh buffer and centrifuged as before, and the final pellet was resuspended in the original volume of ice-cold buffer by homogenization. Binding was assayed in triplicate with 1 nM [^{3}H](*R*)α-methylhistamine. Incubation with [^{3}H](*R*)α-methylhistamine was carried out at 25°C for 60 min, and the reaction was terminated by addition of 5 ml of ice-cold buffer and rapid filtration on a glass fiber filter (GF/B, Whatman, Maidstone, UK). The filter was washed three times with 5 ml of cold buffer, and the radioactivity trapped on the filter was counted in 10 ml of Aquasol 2 with a scintillation counter. Specific binding was defined as the radioactivity bound after subtraction of nonspecific binding, determined in the presence of 5 μM thioperamide. Protein concentration was estimated

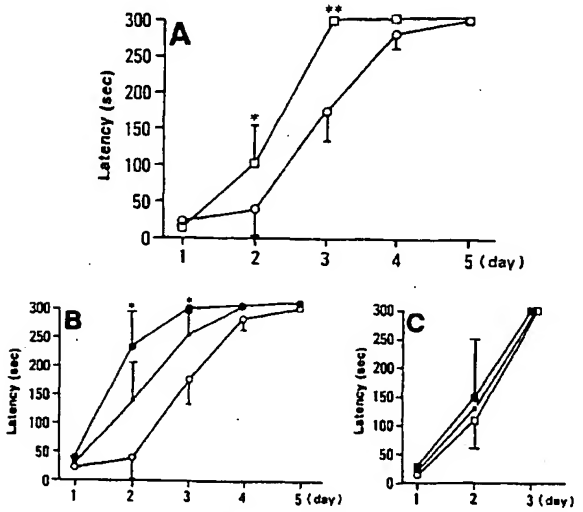

FIG. 1. Passive avoidance experiments in SAM. (A) Mean step-through latencies in SAM-P/8 (○, n = 23) and SAM-R/1 (□, n = 17) mice treated with saline. (B) Mean step-through latencies of SAM-P/8 mice treated with 7.5 (•, n = 11), 15 mg/kg thioperamide (●, n = 12), and saline (○, n = 23) are shown. (C) Mean step-through latencies of SAM-R/1 mice treated with 7.5 (■, n = 11), 15 mg/kg thioperamide (■, n = 13), and saline (□, n = 17) are illustrated. Data are for 5 consecutive days of training. Vertical bars indicate SD. *$p < 0.05$; **$p < 0.01$.

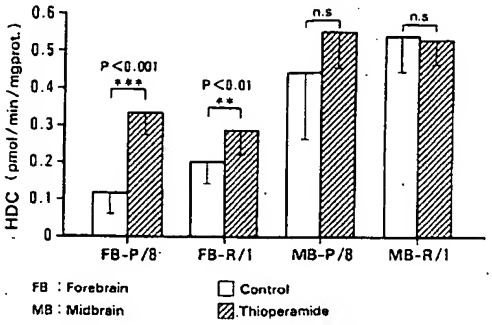

FIG. 2. Histidine decarboxylase (HDC) activities in the forebrains and midbrains of SAM-P/8 and SAM-R/1. The HDC activity in thioperamide-treated mice is shown by hatched columns and that of saline-treated mice by open columns. Vertical bars indicate SD. $^{**}p < 0.01$; $^{***}p < 0.001$. NS, not significant.

by the method of Bradford using bovine serum albumin as a standard (BIO-RAD protein assay kit).

Chemicals

[^{3}H](R)α-Methylhistamine (39 Ci/mmol) and thioperamide maleate were generous gifts from Green Cross Corporation (Osaka, Japan) and Sumitomo Pharmaceut. Co (Osaka, Japan), respectively. Other chemicals were of reagent purity and were obtained commercially.

Statistics

The significance of differences in latency of passive avoidance and HDC activity was determined by analysis of variance (ANOVA) followed by Duncan's test.

RESULTS

The mean step-through latency in a passive avoidance test was measured for the aged SAM-P/8 and SAM-R/1 groups treated with 7.5, 15 mg/kg of thioperamide or saline. As shown in Fig. 1, there was no significant difference in the step-through latencies of any groups on the first day, which was the day of training. In acquisition trials, SAM-P/8 treated with saline showed a shorter step-through latency than saline-treated SAM-R/1 (Fig. 1A). Intraperitoneal administration of thioperamide at a dose of 15 mg/kg resulted in significant prolongation of the response latency in SAM-P/8 mice, which showed a marked age-accelerated deterioration in learning tasks of passive avoidance (Fig. 1B). Differences between the SAM-P/8 mice treated with 15 mg/kg thioperamide and saline were observed on the second and third days ($p < 0.05$). In contrast, thioperamide had no significant effect on the response latency of SAM-R/1 at any doses (Fig. 1C). Thioperamide administration at a dose of 7.5 mg/kg slightly improved passive avoidance response latency in SAM-P/8 mice, but the effects were not statistically significant (Fig. 1B).

After the behavioral test, the brain HDC activity of the mice was determined. The mean HDC activities in the brains of SAM treated with 15 mg/kg thioperamide or saline are summarized in Fig. 2. In control mice, the HDC activity of the forebrain of old P/8 mice was significantly lower than that of old R/1 mice ($p < 0.05$). There were no significant differences in the HDC activities of the midbrain of thioperamide- and saline-treated mice in the two strains. However, thioperamide significantly increased the HDC activity in the forebrain of SAM-P/8 ($p < 0.001$) and R/1 ($p < 0.01$) mice. The increase in HDC activity in the forebrain induced by thioperamide was apparently related to the effect of thioperamide on the mean step-through latency.

In mouse forebrain, the maximum binding of [^{3}H](R)α-methylhistamine at 25°C was observed at a concentration of approximately 4-8 nM and the half-maximum binding at about 2 nM. Histamine H_3 receptors were distinctly distributed in cerebral cortex, striatum, substantia nigra, ventral pallidum, and olfactory nucleus. The autoradiographic distributions of the specific [^{3}H](R)α-methylhistamine binding in brains of old P/8 and R/1 mice were similar (data not shown). The distribution in mouse brain was consistent with that in rat and guinea pig brains (2,8). There was no significant difference in the bindings of [^{3}H](R)α-methylhistamine at 1 nM in the forebrains of young and old P/8 and R/1 mice (Table 1).

DISCUSSION

Biochemical and pharmacological lines of evidence indicate that cholinergic dysfunction plays an important role in age-related memory disturbances in humans and animals. A number of neurotransmitter and neuropeptide systems in both cortical and subcortical brain regions are compromised in Alzheimer-type dementia. In most cases of Alzheimer's disease, the cholinergic innervation of the cerebral cortex is degenerated, and in some cases, the noradrenergic and serotonergic innervations are also reduced (15). However, little is known about changes in the histaminergic neuron system in normal and abnormal aging, although ontogenic and developmental changes in the histaminergic neuron system in mammalian brain have been studied. In contrast to the metabolites of other aminergic transmitters, the levels of histamine metabolites are known to increase in human cerebrospinal fluid with age (14). Mazurkiewics-Kwilecki and Nsoswah reported that histamine in the brain normally increases with age, but decreases in Alzheimer's disease (10). On the other hand, Caca-

TABLE 1

[^{3}H](R)α-METHYLHISTAMINE BINDING TO MEMBRANES OF THE FOREBRAINS OF SAM-P/8 AND R/1 MICE

	Young (4-6 weeks)		Old (12-16 months)	
	SAM-P/8	SAM-R/1	SAM-P/8	SAM-R/1
Binding	27.0 ± 4.8	30.4 ± 4.9	25.5 ± 2.8	29.4 ± 4.9

Values are means ± 1 SD for five-six determinations. Binding of [^{3}H](R)α-methylhistamine at 1 nM is expressed as fmol/mg protein (see the Method section).

belos et al. found that its level in the posterior hypothalamus is increased in Alzheimer's disease (3). Immunohistochemical study did not confirm that histamine-immunoreactive neurons in the hypothalamus are altered in Alzheimer's disease (1). To clarify the participation of histamine neurons in abnormal aging, we have used two strategies: a senescence-accelerated mouse (SAM) model in animal experiments (11) and positron emission tomography (PET) with [^{11}C]pyrilamine and [^{11}C]doxepin (potent histamine H_1 receptor antagonists) as probes in human studies (26).

In this study, we examined the effects of thioperamide, a histamine H_3 antagonist, on the response latency times, measured by a passive avoidance test, and HDC activity in a murine model of abnormal aging, SAM-P/8, and a control strain, SAM-R/1. HDC activity is a good marker for the central histaminergic neuron system, as described previously (11). Thioperamide significantly improved the impaired latency times of SAM-P/8 mice to those in the control mice, R/1, at a dose of 15 mg/kg. Sakai et al. (16) reported that locomotor activity of W/W^v mice was about 1.5-fold increased at doses of 10–20 mg/kg (as free base) 60 min after IP injection of thioperamide. Therefore, we chose the two doses of 7.5 and 15 mg/kg in our passive avoidance experiments. The effects of thioperamide on the mean step-through latencies were dose dependent in SAM-P/8 mice as shown in Fig. 1B. Neurochemical analyses at the end of behavioral tasks revealed that the HDC activities in the forebrains of SAM-P/8 and -R/1 were also restored to the levels of young SAM-P/8 and -R/1 mice (11). Thioperamide had no significant effect on HDC activity in the midbrain. This difference in the effects of thioperamide in different brain regions is consistent with the regional distribution of histamine H_3 receptors in the brain (2).

In previous studies, SAM-P/8 mice were found not to show any age-accelerated changes in spatial learning, measured by radial-arm maze performance, or water-filled multiple T-maze and T-maze avoidance, in contrast to a passive avoidance task (6). Moreover, no age-related changes in the concentration of acetylcholine were demonstrated in young and old SAM-P/8 and -R/1 mice (6). These findings suggest that the brain mechanisms involved in spatial learning may be different from those in passive avoidance, and that histamine may be one of the neurotransmitters related to the impairment of latency of SAM-P/8 mice in the passive avoidance test.

The histamine H_3 receptor operates in rodents and humans by inhibiting histamine release and synthesis, and is considered to be autoreceptors expressed in histaminergic terminals as well as perikarya (20,21). However, it is now considered to act as a heteroreceptor in the central and peripheral nervous systems. It also regulates the release of other neurotransmitters such as serotonin, noradrenaline, acetylcholine, and γ-aminobutyric acid (4,5,18,19). Recently, Servos et al. suggested that the sleep–waking and behavioral arousal proposed for histaminergic neuronal function is probably indirect, acting via other neurotransmitter systems (22). Thioperamide is reported to interact with the heme moiety of hemoproteins and bind to non-H_3 receptors in peripheral tissues, in addition to acting through histamine H_3 receptors (9,25). It could be possible that thioperamide affected the memory and learning in SAM via other neuronal systems. Although it is not clear whether the effects of thioperamide observed in this study were direct or indirect on the central histaminergic system, this study suggests that histamine H_3 receptor antagonists could improve learning and memory impairment observed in passive avoidance in parallel with the increase in the locomotor activity. We are now examining the exact mechanisms for the effects of thioperamide on the passive avoidance in SAM-P/8.

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Identification and Pharmacological Characterization of a Series of New 1*H*-4-Substituted-Imidazoyl Histamine H_3 Receptor Ligands

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ABSTRACT

A new series of 1*H*-4-substituted imidazole compounds were synthesized and identified as potent and selective histamine (HA) H_3 receptor ligands. These ligands establish that HA H_3 antagonists exhibit stereoselective and conformational preferences in their binding to the HA H_3 receptor. Structure-activity relationships were determined in vitro by HA H_3 receptor-binding affinities using [^{3}H]N$^\alpha$-methylhistamine and rat cerebral cortical tissue homogenates. Several derivatives containing olefin, amide, and acetylene functional groups were identified as potent HA H_3 receptor ligands. In the olefin series, GT-2227 (4-(6-cyclohexylhex-*cis*-3-enyl)imidazole) was identified as a potent HA H_3 receptor ligand with a K_i of 4.2 ± 0.6 nM, while the *trans* isomer (GT-2228) displayed a reduced potency (K_i = 15.2 ± 2.4 nM). GT-2227 was also found to have excellent central nervous system penetration in an ex vivo binding paradigm (ED_{50} = 0.7 mg/kg i.p.). In the acetylene series, GT-2260 and GT-2286 both exhibited high affinity (K_i = 2.9 ± 0.2 and 0.95 ± 0.3 nM) and excellent central nervous system penetration profiles (ED_{50} = 0.43 and 0.48 mg/kg i.p., respectively). As a prototype for the series, GT-2227 showed high affinity for the human HA H_3 receptor (3.2 nM) and minimal affinity for the human HA H_1 (K_i = 13,407 ± 540 nM) and H_2 (K_i = 4,469 ± 564 nM) receptor subtypes. GT-2227 also showed good selectivity for the HA H_3 receptor over a broad spectrum of other neurotransmitter receptors ($IC_{50} \geq 1$ μM). Furthermore, GT-2227 improved acquisition in a cognitive paradigm without behavioral excitation or effect on spontaneous locomotor activity. In summary, the present studies demonstrate the development of novel HA H_3-selective ligands, and lend support for the use of such agents in the treatment of disorders associated with cognitive or attentional deficits.

Histamine (HA) exerts a neurotransmitter function in the peripheral and central nervous systems through its action at three HA receptor subtypes: H_1, H_2, and H_3 (reviewed by Arrang, 1994; Ruat et al., 1994). The HA H_3 receptor is highly localized in the central nervous system (CNS), and low levels are seen in peripheral tissues (Korte et al., 1990). Autoradiographic studies have described the detailed heterogeneous distribution of CNS HA H_3 receptors within brain regions (Pollard et al., 1993). HA H_3 receptors are present on histaminergic nerve terminals in the brains of rats and humans (Arrang et al., 1983, 1988). The HA H_3 autoreceptor modulates the amount of HA synthesized and released from histaminergic neurons (Arrang et al., 1983, 1987a). Furthermore, HA H_3 receptors are present as heteroreceptors in other neurotransmitter systems. The release of serotonin (Fink et al., 1990), dopamine (Schlicker et al., 1993), acetylcholine (Clapham and Kilpatrick, 1992), norepinephrine (Molderings et al., 1992), and Substance P (Taylor and Kilpatrick, 1992) is attenuated by HA H_3 receptor agonists. Conversely, HA H_3 receptor antagonists block these receptors, resulting in increased neurotransmitter release. Theoretically, the use of HA H_3 antagonists may provide for a clinically relevant enhancement of neurotransmitter release and thus provide effective therapies in the treatment of a number of CNS disorders (Timmerman, 1990; Leurs et al., 1998).

Selective pharmacological tools have been developed for each of the HA receptor subtypes, and selective HA H_3 antagonists such as GT-2016, thioperamide, and clobenpropit (VUF-9153) (Fig. 1) have been described (Arrang et al., 1987b; Van der Goot et al., 1992; Tedford et al., 1995; reviewed by Leurs et al., 1995; Stark et al., 1996). However, several structural features of HA H_3 receptor antagonists have yet to be fully exploited. For example, although a stereoselective bias in the binding of HA H_3 agonists has been demonstrated for several potent compounds (i.e., (*R*)-α-meth-

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ABBREVIATIONS: HA, histamine; CNS, central nervous system; [^{3}H]NAMHA, [^{3}H]N$^\alpha$-methylhistamine; SAR, structure-activity relationship; PAR, passive avoidance response; SLA, spontaneous locomotor activity.

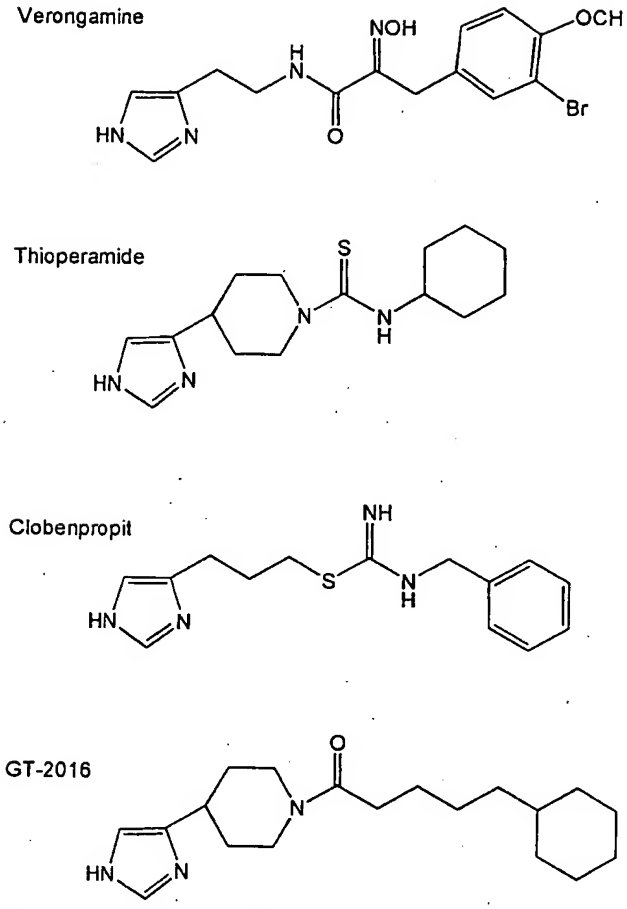


Fig. 1. Structures of verongamine, thioperamide, clobenpropit, and GT-2016.

ylhistamine and (*R*)-α, (*S*)-β–dimethyhistamine), such a relationship has yet to be demonstrated for HA H_3 antagonists. Furthermore, variations in heteroatom substitution have been previously investigated through the incorporation of a number of linker moieties, including amide-, guanidine- thiourea-, isothiourea-, carbamate- and ether-containing substitutions (Arrang et al., 1987b; Van der Goot et al., 1992; Schunack and Stark, 1994; Tedford et al., 1995; Brown et al., 1996; Schlicker et al., 1996). The absence of such heteroatom substitution through the incorporation of an unsaturated carbon spacer has not previously been considered in the development of HA H_3 receptor antagonists.

In addition to the compounds discussed above, the natural product verongamine (Fig. 1), isolated from the marine sponge *Verongula gigantea*, was determined to have moderate HA H_3 receptor affinity with an IC_{50} of 0.5 μM for guinea pig CNS HA H_3 receptors (Mierzwa et al., 1994). Analysis of the structural features of verongamine from a conformational and stereochemical view point provided us with conceptual insight that led to the development of several new series of HA H_3 receptor ligands with high affinity and receptor selectivity. Structure-activity relationship (SAR) studies focused on several structural features illustrated by the derivative shown in Fig. 2. These features include the imidazole ring (A), ethylene bridge (B), amide bond or replacements (C), chirality and substitution of the amino constituent

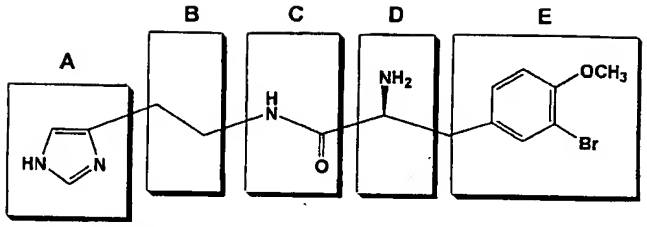


Fig. 2. Model template for HA H_3 receptor ligand development.

(D), and the terminal hydrophobic region (E). Evaluation of these specific elements has led to the development of new, potent, and selective HA H_3 receptor ligands. Herein, the in vitro and in vivo binding characteristics of a variety of newly developed compounds are described (see Tedford et al., 1999). Moreover, the structural requirements for ligand interaction with the HA H_3 receptor and initial pharmacological profiles of selective HA H_3 receptor ligands are discussed.

Materials and Methods

Chemicals. GT compounds and thioperamide were synthesized by Gliatech chemists (Ali et al., 1998, 1999). Other reagents were purchased as indicated: triprolidine (Research Biochemicals International, Natick, MA), aminopotentidine (Tocris Cookson, Bristol, UK), [^{3}H]N$^\alpha$-methylhistamine ([^{3}H]NAMHA; 81.5 Ci/mmol), [^{3}H]pyrilamine (>20 Ci/mmol; DuPont NEN Research Products, Boston, MA), and [^{125}I]iodoaminopotentidine (2000 Ci/mmol; Amersham, Arlington Heights, IL or provided by Drs. Leurs and Timmerman at the Leiden/Amsterdam Center for Drug Research, the Netherlands). Clobenpropit was kindly provided by Dr. Timmerman.

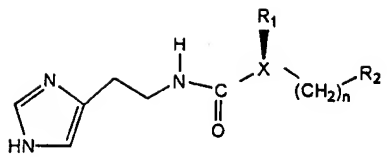
Animals. Male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN) and housed two per cage on a 12-h light/dark schedule with ad libitum access to Teklad Mouse/Rat Diet 7012 (Harlan Laboratories) and water in accordance with the Animal Welfare Act of 1994 and amendments. Animals were acclimated to laboratory conditions for a minimum of 1 week before tissue harvesting.

In Vitro HA H_3 Receptor-Binding Analysis. HA H_3 receptor affinity was determined in rat cerebral cortical membranes with [^{3}H]NAMHA as described previously (Tedford et al., 1995). Animals were euthanized by rapid decapitation and cerebral cortical tissues were harvested and frozen on dry ice. Cerebral cortical membranes were prepared in 50 mM sodium-PBS (pH 7.5 at 4°C) containing EDTA (10 mM), phenylmethylsulfonyl fluoride (0.1 mM), chymostatin, and leupeptin (each 0.2 mg/50 ml). The final membrane pellets were resuspended in water and stored frozen at −80°C before use. Protein concentrations were determined using the Coomassie Plus Protein Assay (Pierce, Rockford, IL).

Competition binding was carried out in a total volume of 0.2 ml of 50 mM sodium-phosphate buffer (pH 7.4) using ~1 nM [^{3}H]NAMHA and 0.003 to 10,000 nM concentrations of the test compounds. Nonspecific binding was determined using 10 μM thioperamide. Samples were incubated for 40 min at 25°C and subsequently filtered through Whatman GF/C glass fiber filters presoaked in binding buffer with 0.3% polyethyleneimine using an Inotech cell harvester (Inotech Biosystems International, Lansing, MI). The filters were rapidly washed three times with Tris-NaCl buffer (25 and 145 mM, respectively, pH 7.4, 4°C). Samples were quantitated using Ecolume scintillation cocktail (ICN Biomedicals, Costa Mesa, CA) and a Packard model 1900TR liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL). IC_{50} values were extrapolated from a plot of receptor occupancy (i.e., percent bound) versus log [competitor]. Inhibition constants (K_is) were determined using the equation: $K_i = IC_{50}/(1 + ([\text{ligand}]/[K_d])$, where K_d = 0.4 nM for [^{3}H]NAMHA.

The affinity of GT-2227 for the human HA H_3 receptor was also

TABLE 1
Histamine H_3 receptor-binding affinities



GT	X	R_1	n	R_2	H_3 (K_i, nM)
2130	CH	(*L*)-NH_2	1		104 ± 14
2136	CH	(*D*)-NH_2	1		2814 ± 112
2140	CH	(*L*)-NH_2	1		31 ± 2
2142	CH	MeNH	1		1593 ± 186
2146	CH	(*L*)-NH_2	0		630 ± 51
2148	CH	(*L*)-NH_2	2		11 ± 2
2174	CH	H	2		16 (n=1)

K_i values are mean ± S.E. of three individual experiments unless otherwise indicated.

evaluated. In these studies, membranes were prepared from a single sample of postmortem human forebrain tissue using the technique described above for rat brain tissue. Human tissue was provided by Dr. E. Stopa (Brown University School of Medicine, Providence, RI). Binding was conducted and samples were quantitated as described for the rat HA H_3 receptor binding.

Ex Vivo HA H_3 Receptor-Binding Analysis. Ex vivo HA H_3 receptor occupancy was determined in rat cerebral cortical membranes with [^{3}H]NAMHA as described previously (Taylor et al., 1992; Tedford et al., 1995). Rats were acutely treated with a single i.p. injection of compound (n = 4/group). Drugs were administered in a final volume of 1 ml/kg. The animals were euthanized by a lethal injection of sodium pentobarbital (150 mg/kg i.p., Nembutal; Abbott Laboratories, North Chicago, IL) at the indicated times postadministration. Following euthanasia, the upper torso was transcardially perfused through the aortic arch with 60 ml of 0.9% saline to remove potential vascular drug contamination. Brains were removed, dissected, and frozen on dry ice. The tissue was stored at −80°C before conducting the binding studies. On the day of binding experiments, the tissue was homogenized using a motor-driven tissue grinder (Omni 1000) in 9 volumes (w/v) of 50 mM sodium-phosphate buffer (pH 7.4). Ex vivo binding was carried out in a total volume of 0.4 ml of 50 mM sodium-phosphate buffer containing ~1 nM [^{3}H]NAMHA and 0.15 to 1 mg of protein. Nonspecific binding was determined using 10 μM thioperamide. Samples were treated as described above for the in vitro binding. ED_{50} values (doses that produced 50% inhibition of [^{3}H]NAMHA binding) in milligrams per kilograms were determined by linear regression analysis of the data on a log-linear plot.

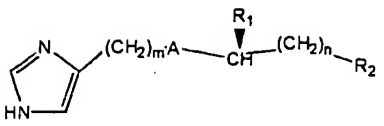
In Vitro Human HA H_1 and H_2 Receptor-Binding Analysis. Before binding, human HA H_1 or H_2 receptor-expressing cells were harvested, washed, and stored as a pellet at −80°C. Stable human HA H_1 receptor-expressing Chinese hamster ovary cells were provided by Drs. Leurs and Timmerman (Leiden/Amsterdam Center for Drug Research). The HA H_1 receptor was expressed at ~1 pmol/mg protein in these cells. In preparation for binding, cell cultures were expanded and homogenates were prepared by sonication in water. HA H_1 receptor affinity was determined using membranes prepared from HA H_1 receptor-transfected cells and [^{3}H]pyrilamine (Tedford et al., 1995). Binding was performed in a total volume of 0.4 ml of 50 mM sodium/potassium-phosphate buffer containing 1 mM $MgCl_2$ (pH 7.4, 25°C). Nonspecific binding was determined in the presence of 10 μM triprolidine. For competition studies, 4 nM [^{3}H]pyrilamine was incubated with ~8 μg of membrane protein for 30 min at 25°C in polypropylene tubes with increasing concentrations of test compounds. Binding was terminated and samples were quantitated as described for HA H_3 receptor binding.

HA H_2 receptor affinity was determined using membranes prepared from HA H_2 receptor-transfected Chinese hamster ovary cells and ^{125}[I]iodoaminopotentidine. One HA H_2 clone expressing ~2 pmol/mg protein was expanded for use in receptor-binding assays. Membranes were prepared as described above. Binding was performed in a total volume of 0.1 ml of 50 mM sodium/potassium-phosphate buffer (pH 7.4, 25°C). Nonspecific binding was determined in the presence of 10 μM tiotidine. For competition studies, 0.25 nM ^{125}[I]iodoaminopotentidine was incubated with ~7.5 μg of membrane protein for 2.5 h at 25°C in polypropylene tubes with increasing concentrations of test compounds. Binding was terminated and samples were quantitated as described for HA H_3 receptor binding.

GT-2227 was also evaluated in a CNS receptor profile screen (Quintiles, Edinburgh, UK) to determine affinity for a number of non-HA receptors and ion channels. A single concentration (1.0 μM) of GT-2227 was tested in triplicate in a single experiment and the percentage of inhibition was determined.

Spontaneous Locomotor Activity. Spontaneous locomotor activity (SLA) was measured in adult Sprague-Dawley rats (200–300 g) using automated Omnitech digiscan animal activity monitors (42.5 × 42.5 × 40 cm; Omnitech, Columbus OH) between 9:00 AM

TABLE 2
Histamine H_3 receptor-binding affinities



GT	A	R_1	m	n	R_2	H_3, (K_i, nM)
2227	H, C=C, H	H	2	1		4.2 ± 0.6
2228	H, C=C, H	H	2	1		15.2 ± 2.4
2231	H, C=C, H	(L)-NH_2	2	1		1.0 ± 0.1
2260	—C≡C—	H	2	1		2.9 ± 0.2
2286	—C≡C—	H	2	1		0.95 ± 0.3
2287	—C≡C—	H	2	2		3.4 ± 1.7
2288	—C≡C—	H	0	2		1029 ± 27
2293	—C≡C—	H	2	1		0.83 ± 0.04
2321	$-CH_2-CH_2-$	H	2	1		75 (n=2)
2327	H, C=C, H	H	2	1		5.5 ± 0.02

K_i values are mean ± S.E. of three individual experiments unless otherwise indicated.

and 2:00 PM (lights out). Data were collected for the following parameters: total, horizontal, central and peripheral activities, stereotypes, and vertical movements. Total counts, number of occurrences, and total time involved in each behavior were determined. Thirty-minute baseline readings (5-min collection intervals) were collected, after which the animals were administered drug or vehicle. Following treatment, an additional 90 min of activity monitoring was conducted.

Passive Avoidance Training and Drug Treatment Studies. Postnatal day 15 to 16 (P15–16) rat pups were used for the 10-trial passive avoidance response (PAR) testing. Animals were singly placed in a Coulbourn Instruments (Allentown, PA) small animal shuttle box. The shuttle box has a lighted compartment divided by a door leading to a dark compartment. The animals were placed facing away from the dark compartment and the time to turn and enter the dark compartment was measured (latency). Upon entering the dark compartment, the animal trips an automated door closure and, after a 5-s delay, a mild foot shock (0.5 mA, 60 Hz, 2 s) is delivered through a metal grid floor. Immediately after the shock, the pup is removed and returned to its home cage for 3 min. Animals were returned to the lighted compartment after 3 min and the step-through latency (maximum of 180 s) was recorded for a total of 10 trials.

For drug treatment studies, P15 to 16 rat litters (10–12 pups each) were randomly divided into vehicle- or drug-treated groups. Each individual litter was assessed for general developmental traits (i.e., weight, physical appearance, visual activity). Variation in litter size, weight, physical appearance, and so forth was assessed throughout the experiments and noted. Results from many litters indicated that P15 to 16 litters were comparable in development and physical appearance. Each dose of test compound was evaluated with an equivalent number of vehicle-treated littermate controls on a given experimental day. Approximately 2 to 3 litters were used for each dose of test compound (n = 12–18/group). Test compounds were administered 30 min before PAR testing.

Statistical Analysis. PAR statistical analysis was restricted to comparisons between the drug-treated animals and their vehicle-treated littermate controls for a particular dose of drug. Both the median and mean latencies (seconds) were determined for each trial. Statistically significant differences ($p < .05$) for a given trial were determined using a nonparametric Kruskal-Wallis test. Mean latencies ± S.E. are depicted for graphical representation.

Results

In Vitro HA H_3 Receptor-Binding and SAR Studies. The initial synthesis of several HA amides provided several key SAR for the development of a series of potent HA H_3 receptor ligands (Table 1). The amide resulting from the coupling of HA with (*L*)-phenylalanine afforded a compound, GT-2130, with good HA H_3 receptor affinity (K_i = 104 nM). In contrast, the (*D*)-phenylalanine analog exhibited a marked reduction in HA H_3 receptor affinity (K_i = 2,814 nM). This gave direct evidence for a stereoselective bias for the HA H_3

TABLE 3
Human HA receptor selectivity profile for GT-2227

Compound	H_1 (K_i, nM)[a]	H_2 (K_i, nM)[b]	H_3 (K_i, nM)[c]	H_1/H_3 ratio[d]	H_2/H_3 ratio[d]
GT-2227	13,407 ± 540	4,469 ± 564	3.2	4,190	1,397

[a,b] The affinity of GT-2227 for the human HA H_1 and H_2 receptors, respectively, was determined using Chinese hamster ovary cells expressing the respective HA receptor subtype (mean ± S.E., n = 4).
[c] The affinity of GT-2227 for the human HA H_3 receptor was determined using membranes prepared from postmortem human forebrain tissue (n = 1).
[d] Ratios were calculated by dividing the HA H_1 or H_2 K_i values by the HA H_3 K_i value.

TABLE 4
Receptor screening analysis for GT-2227

Binding Site	% Inhibition[a]	Binding Site	% Inhibition[a]
Adenosine A_1	−2.8	Imidazoline (I_2)	63.8
Adenosine A_2	−41.6	K^+_{ATP} channel	
Adrenergic		Central	−0.9
$\alpha_{1\,(nonselective)}$	32.5	Peripheral	−60.9
$\alpha_{2\,(nonselective)}$	75.1	Cholinergic	
β_1	−2.1	M_1	11.4
β_2	−17.9	M_2	−7.6
Benzodiazepine		M_3	11.4
Central	−20.0	Nicotine$_{(CNS)}$	−3.7
Peripheral	−14.5	Na^+ channel	28.1
Ca^{2+} channel (L)	0.3	Opioid	
Dopaminergic		μ	−6.5
D1	−13.0	κ	−13.1
D2	14.5	δ	−9.2
Endothelin		Serotonergic	
ET_A	−2.2	5-$HT_{1\,(nonselective)}$	5.4
ET_B	−6.7	5-HT_{1A}	51.3
Glutamate	1.8	5-HT_{1B}	−4.0
NMDA	−36.2	5-HT_{1D}	23.4
AMPA	−25.1	5-HT_{2A}	0.0
Kainate	−38.2	5-HT_{2C}	5.7
γ-Aminobutyric acid	−32.8	5-HT_3	6.4

[a] Total and nonspecific binding as well as the percentage of inhibition by GT-2227 (1 μM) for the given receptor/ion channel were determined. Values represent the mean of triplicate determinations in a single experiment.

receptor and illustrated the importance of the chiral amino center for enhancing HA H_3 receptor affinity. Elongation of the carbon tail (GT-2148 versus GT-2130) and/or replacement of the terminal phenyl group with a cyclohexyl moiety (GT-2130 versus GT- 2140) provided additional optimization of HA H_3 affinity within the amide series. Substitution on the NH_2 group (GT-2142) resulted in significant loss in HA H_3 receptor affinity, suggesting the importance of the primary chiral amino group. In addition, analogs of compounds without the primary amino group (e.g., GT-2174 versus GT-2140) also demonstrated potent HA H_3 receptor activity, provided that optimization of the carbon chain length and hydrophobic tail were made. Moreover, incorporation of the chiral NH_2 group into various conformationally restricted heterocyclic ring systems (data not shown) indicated that an octahydroindole moiety was an acceptable substitution.

Table 2 summarizes data for several olefin and acetylene analogs of the amide derivatives. Potent HA H_3 affinity was seen in the *cis*-olefin derivative GT-2227, whereas reduced affinity was seen with the *trans*-olefin isomer GT-2228, demonstrating a preference for the *cis*-olefin geometry. GT-2231, which predominantly contains the *trans*-olefin configuration and the chiral amino substituent, demonstrated high affinity binding (K_i = 1 nM). A series of acetylene derivatives were prepared using a Topliss operational scheme (Topliss, 1972) for aliphatic side chain substitution. The most potent compounds of the acetylene series were GT-2286 and GT-2293, with affinities in the subnanomolar range (0.95 and 0.83 nM, respectively). GT-2321, which contains the analogous saturated hydrocarbon chain, exhibited dramatically reduced HA H_3 affinity as compared with the olefin and acetylene compounds (GT-2227 and GT-2260, respectively).

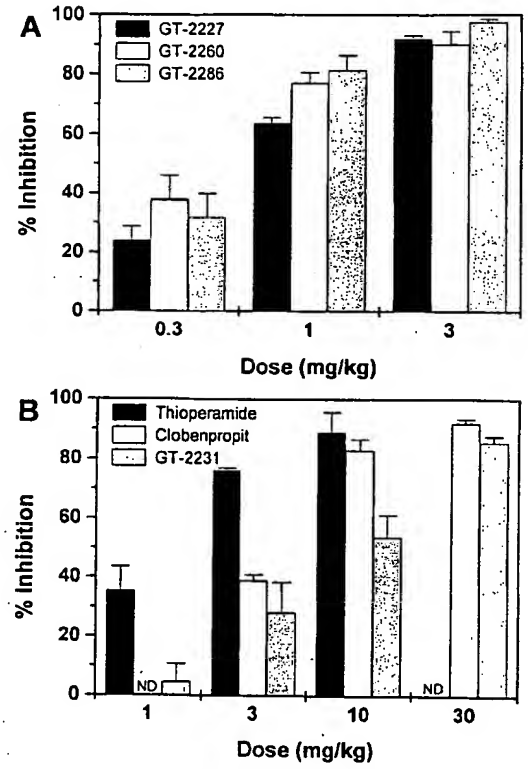


Fig. 3. Ex vivo HA H_3 receptor-binding profile for selected ligands in rat brain cortex. A, GT-2227, GT-2260, and GT-2286 (0.3, 1, and 3 mg/kg) and the respective vehicles were administered i.p. 1 h before the animals were euthanized. Ex vivo HA H_3 receptor binding was determined for each animal (n = 3–4/group) in femtomoles per milligram of protein and expressed as a percentage of values from vehicle-treated animals. Absolute values for vehicle-dosed animals were 38.00 ± 2.07, 27.76 ± 0.88, and 31.10 ± 0.92 fmol/mg protein for GT-2227, GT-2260, and GT-2286, respectively (mean ± S. E., n = 4). B, thioperamide (1, 3, and 10 mg/kg), clobenpropit (3, 10, and 30 mg/kg), and GT-2231 (1, 3, 10, and 30 mg/kg) and the respective vehicles were administered i.p. 1 h before the animals were euthanized. Ex vivo HA H_3 receptor binding was determined for each animal (n = 3–4/group) in fmol/mg protein and expressed as a percentage of values from vehicle-treated animals. Absolute values for vehicle-dosed animals were 30.75 ± 1.9, 29.47 ± 6.99, and 31.87 ± 2.34 fmol/mg protein for thioperamide, clobenpropit, and GT-2231, respectively (mean ± S. E., n = 4). ND, not determined.

Receptor Selectivity Profile. The HA receptor selectivity profile was evaluated for one of the more potent olefin-containing analogs, GT-2227. GT-2227 had minimal affinity for the human HA H_1 and H_2 receptor subtypes (Table 3). The binding of GT-2227 to human HA H_3 receptors was evaluated using membranes prepared from human forebrain autopsy tissue. These affinities provided receptor selectivity ratios of 4190 and 1397 for the human HA H_3 receptor with

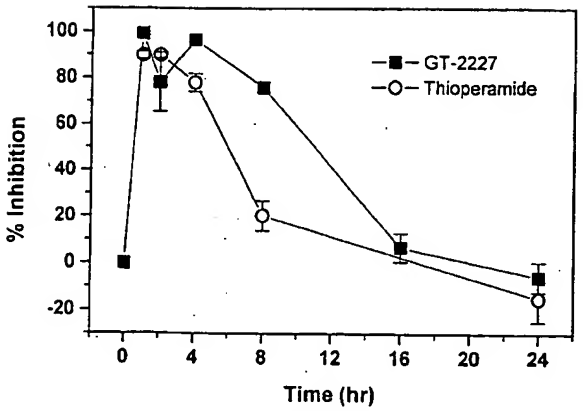


Fig. 4. Twenety-four-hour time course ex vivo binding profiles for GT-2227 and thioperamide in rat brain cortex. GT-2227 or thioperamide (10 mg/kg) and vehicle were administered i.p. Animals were euthanized at 1, 2, 4, 8, 16, or 24 h after administration of drug. Ex vivo HA H_3 receptor-binding was determined for each animal (n = 4/group) in femtomoles per milligram of protein and expressed as percentage of vehicle-treated values. Absolute values for vehicle-dosed animals were 33.12 ± 3.36 and 37.65 ± 3.74 fmol/mg protein for GT-2227 and thioperamide, respectively (mean ± S. E., n = 4).

respect to the human HA H_1 and H_2 receptors, respectively. A general receptor screening profile for GT-2227 was also performed for a variety of receptors and ion channels. Overall, 1 μM GT-2227 produced minimal inhibition of ligand binding in these assays (Table 4). This concentration of GT-2227 did, however, produce >50% inhibition of ligand binding to α_2 and I_2 receptors in preliminary binding studies (Table 4). More detailed analysis of binding to these receptors provided IC_{50} values of 1274 and 1342 nM for α_2 and I_2 receptors, respectively.

Ex Vivo HA H_3 Receptor-Binding Studies. The CNS penetration for several of the more potent HA H_3 ligands was evaluated using the ex vivo binding technique. Rats were dosed with 0.3 to 30 mg/kg (i.p.) compound and, after 1 h, the rats were euthanized and the penetration of compound into the CNS was evaluated. Following 0.3, 1, and 3 mg/kg (i.p.), three of the most potent GT compounds, GT-2227, GT-2260, and GT-2286, produced dose-dependent inhibition of [^{3}H]NAMHA binding in cerebral cortical homogenates prepared from drug- versus vehicle-treated animals (Fig. 3A). Each of the three compounds produced very similar inhibition profiles, with 25 to 35% inhibition of [^{3}H]NAMHA binding at 0.3 mg/kg and near maximal receptor occupancy at the 3-mg/kg doses 1 h postadministration. Log-linear regression analysis of these data provided similar ED_{50} values of 0.7 ± 0.05, 0.43 ± 0.12, and 0.48 ± 0.14 mg/kg (mean ± S.E.) for GT-2227, GT-2260, and GT-2286, respectively. These compounds represent some of the most potent, CNS available HA H_3 ligands developed to date. The ex vivo binding profile for GT-2227 in postnatal day 16 rat pups was also evaluated. Pups were dosed with 0.3, 1, and 3 mg/kg GT-2227 (i.p.) for 1 h. The ED_{50} was 0.49 ± 0.04 mg/kg (mean ± S.E.) and is similar to what was observed in adult rats. Two known HA H_3 antagonists, thioperamide and clobenpropit, were also compared for CNS penetration. Thioperamide produced a dose-dependent inhibition of [^{3}H]NAMHA binding at 1, 3, and 10 mg/kg (i.p.), as well as clobenpropit at 3, 10, and 30 mg/kg (i.p.) (Fig. 3). The ED_{50} values for these compounds

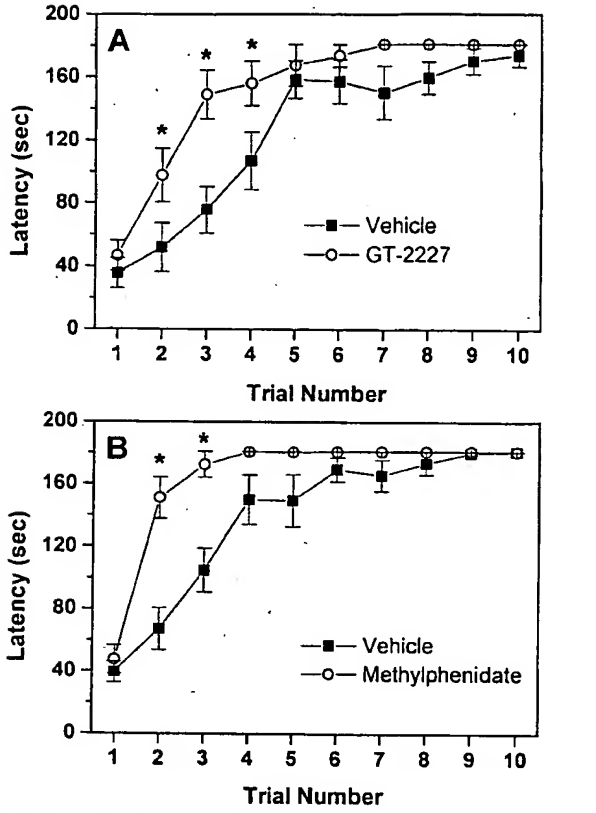


Fig. 5. Effect of GT-2227 and methylphenidate on acquisition of a 10-trial PAR in rat pups. P15 to 16 rat pups were tested for acquisition of a multitrial PAR with a 3-min intertrial period. Littermates were equally divided into vehicle- or drug-treatment groups. GT-2227 (A) or methylphenidate (B) was administered i.p. at a dose of 1 or 3 mg/kg, respectively, 30 min before training. Animals were returned to their home cage with their littermates for the intertrial time period. Data are expressed as mean latency ± S. E. (n = 12–18/group). *Indicates statistically significant differences ($p < .05$) between drug- and vehicle-treatment groups at the specific trial number. Nonparametric statistical analysis (Kruskal-Wallis test) was conducted on median latencies (seconds).

were 1.49 ± 0.64 and 3.88 ± 4.41 mg/kg (mean ± S.E.), respectively.

GT-2231 was also evaluated for CNS penetration. Rats were treated with 1 to 30 mg/kg GT-2231 (i.p.), and CNS penetration was evaluated after 1 h. Although the in vitro binding affinity of GT-2231 is similar to that of GT-2227 (Table 2), the ex vivo binding profile for GT-2231 was shifted lower by an order of magnitude. This decrease in CNS penetration for GT-2231 is reflected in the ED_{50} value of 7.39 ± 7.1 mg/kg (mean ± S.E.), and suggests that in vivo protonation of the NH_2 group may restrict CNS penetration. In addition, the ex vivo binding profile for one of the amides, GT-2140, was also evaluated at 3, 10, and 30 mg/kg (i.p.). This compound showed poor CNS availability with an ED_{50} in excess of 30 mg/kg (data not shown). These results are likely to be related to the amide linkage, which could be anticipated to undergo proteolytic hydrolysis, resulting in reduced bioavailability, and a compromise of CNS penetration.

The 24-h time course of CNS penetration and HA H_3 receptor occupancy were also evaluated for GT-2227 and thioperamide (10 mg/kg each i.p.). Both GT-2227 and thioperamide produced near maximal inhibition of [^{3}H]NAMHA

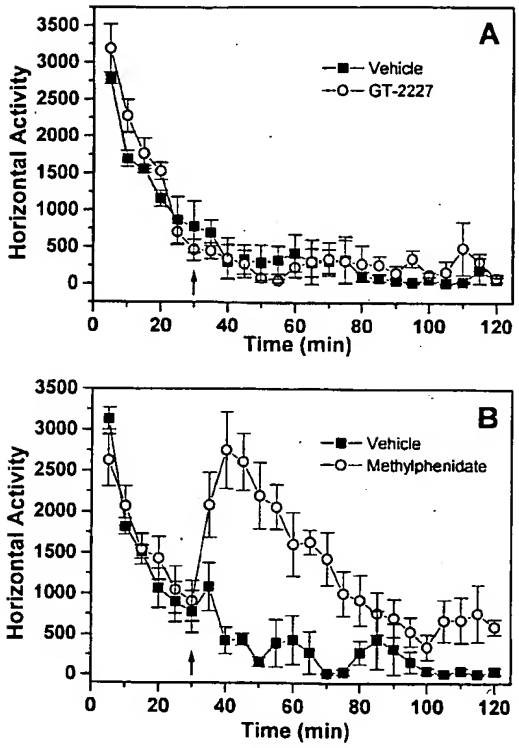


Fig. 6. Effect of GT-2227 and methylphenidate on SLA in adult rats. Adult rat (>30 days) SLA patterns were measured every 5 min for a total of 2 h. GT-2227 or methylphenidate (1, 3, or 10 mg/kg, i.p.) was administered after a 30-min baseline SLA was established. Group mean horizontal activity measurements (beam breaks) are presented for the 10-mg/kg dose of GT-2227 in A and for the 3-mg/kg dose of methylphenidate in B, as well as the respective vehicle-treated groups (mean ± S. E., n = 12–18/group).

binding within 1 h of i.p. administration (Fig. 4). High levels of receptor occupancy were maintained out to 8 h for GT-2227, while receptor occupancy for thioperamide was down to 20% at the same time point. Receptor occupancy returned to normal within 16 to 24 h following the single acute administration of GT-2227 and within 8 to 24 h following administration of thioperamide. Oral administration of GT-2227 (10 mg/kg) produced a CNS HA H_3 receptor occupancy time course profile essentially identical with that of the i.p. (10 mg/kg) administration, with high levels of receptor occupancy maintained out to 8 h following a single acute administration (data not shown).

Effects of GT-2227 and Methylphenidate on Cognitive Deficits in Juvenile Rat Pups. Cognitive impairments have been described in developing rat pups that display rapid development of normal cognitive capabilities at approximately 3 weeks after birth (Duméry and Blozovski, 1987). This immature rat model can be used to assess whether compounds may enhance learning and memory, without the conflicting use of neurotoxins, lesions, or amnesic agents to induce a cognitive deficit. We tested juvenile rat pups at various postnatal times for cognitive function in a single-trial PAR. Initially, 48-h retention latencies (seconds) were determined in juvenile rat pups at P15 to 35 following a single-trial PAR. Deficits in the 48-h recall of a single-trial PAR test were seen in the P15 to 16 rat pups (data not shown).

The rate of learning was subsequently examined in a single-day 10-trial PAR paradigm to evaluate learning capabilities of the juvenile rat pups (P15–16). Acquisition of the multitrial PAR was complete by trial 10; however, clear sustained attentional or learning deficits were apparent in the rat pups (Fig. 5, vehicle). Treatment of pups with GT-2227 resulted in an enhanced acquisition rate versus their vehicle-treated littermates as suggested by the increase in step-through latencies seen at trials 2, 3, and 4 (Fig. 5A). Improvements were evident by trial 2 and acquisition of the task was maximal by trial 4. Similar significant improvements were also seen with a 3 mg/kg (i.p.) dose of GT-2227 (data not shown). Methylphenidate, a known CNS stimulant with cognitive and vigilance enhancing properties, was also tested in single-day 10-trial PAR paradigm. Methylphenidate was administered 30 min before acquisition training in the multitrial PAR test at 3 mg/kg (i.p.). Treatment of pups with methylphenidate resulted in an enhancement of acquisition rate versus their vehicle-treated littermates (Fig. 5B) similar to what was seen with GT-2227. Improvements following methylphenidate administration were evident by trial 2 and acquisition of the task was maximal by trial 3 to 4. There were no differences between GT-2227- and methylphenidate-treated groups versus their respective vehicle-treated groups in entry times at trial 1, indicating that neither compound affected motor performance. GT-2227 was also evaluated in the single-day 10-trial PAR paradigm using a subthreshold foot shock. In these studies, vehicle-treated pups did not learn to avoid the shock over the 10-trial testing period and GT-2227 did not provide any enhancement of learning (data not shown). These data suggest that the effectiveness of GT-2227 did not result from enhanced sensitivity to the foot shock in the PAR. In summary, these studies indicate that HA H_3 receptor blockade may provide improvements in arousal or attentional aspects and enhance acquisition in these developmentally immature animals.

Effects of GT-2227 and Methylphenidate on SLA in Adult Rats. HA H_3 receptor blockade may result in increased CNS HA release and has been shown to induce electroencephalogram activation or arousal (Lin et al., 1990; Monti et al., 1991). Previous studies have not demonstrated profound psychomotor stimulant properties for HA H_3 antagonists, compared to known psychomotor stimulants such as methylphenidate. GT-2227 (1, 3, and 10 mg/kg i.p.) was tested in adult rats for potential psychomotor stimulant effects on SLA. The doses tested were chosen to provide for 50 to 100% HA H_3 cerebral cortical receptor blockade and sustained duration. Normal habituation to the novel environment was evident before either vehicle or drug treatment in the adult rats (>30 days old). Thirty minutes after exposure to the activity chambers, animals (n = 12–18/group) were administered GT-2227 or methylphenidate and SLA was recorded for the next 90 min. GT-2227 had no effect on SLA at any of the tested doses in adult rats as measured by horizontal activity (Fig. 6A) as well as total, central, and peripheral cage activities, stereotypes, or vertical movements (data not shown). Methylphenidate (1, 3, and 10 mg/kg i.p.), however, produced a dose-dependent increase in SLA as measured by horizontal activity. This psychomotor stimulation induced by methylphenidate was dose-dependent (data not shown), and the effect of the 3-mg/kg dose was maintained for approximately 1 h (Fig. 6B).

Discussion

In the present studies, a new series of 1*H*-4-substituted-imidazoyl HA H_3 receptor ligands were synthesized and tested for HA H_3 receptor affinity and selectivity. The development of this series of HA H_3 ligands was conceptually derived using the natural product verongamine (Mierzwa et al., 1994) as a synthetic template. Several structural activity relationships were demonstrated that provide new insights into the binding characteristics of the HA H_3 receptor. Importantly, the chiral amino functionality provided evidence that the HA H_3 receptor exhibits a stereoselective bias in antagonist ligand-binding interactions. This has been demonstrated previously with several HA H_3 agonists [i.e., (*R*)-α-methylhistamine and (*R*)-α, (*S*)-β–dimethyhistamine], but had not been evaluated for HA H_3 antagonists. Additionally, although amide-, guanidine- thiourea-, isothiourea-, carbamate-, and ether-containing series of HA H_3 receptor antagonists have been described (Arrang et al., 1987b; Van der Goot et al., 1992; Schunack and Stark, 1994; Tedford et al., 1995; Brown et al., 1996; Schlicker et al., 1996), the incorporation of a three to four unsaturated carbon spacer (Fig. 2C) had not previously been disclosed in the development of potent HA H_3 receptor antagonists. Clearly, the present studies provide support for the use of acetylene and olefin moieties as spacer groups. Most surprising is that potent HA H_3 receptor affinity was maintained despite the removal of all heteroatoms which would transcend into the binding domain associated with the terminal amino group of HA.

Molecular modeling efforts further support our data and suggest that a nonpolar, planar spacer (olefin and acetylene functionalities) can effectively be used as equivalents to the polar and planar amide or amide-oxime spacer in verongamine (Ali et al., 1998). However, it is clear from these studies that the imidazole head, as well as the position, orientation, and chemical makeup of the middle spacer and terminal hydrophobic pieces must act harmoniously to provide a preferred conformation for interaction with the HA H_3 receptor. The orientation of these structural features is illustrated by the enhanced affinity of the *cis*-olefin versus the *trans*-olefin isomer, GT-2227 versus GT-2228.

The use of a template which systematically explored the various structural features required for HA H_3 receptor activity has led to the development of new compounds which maintain high HA H_3 receptor affinity and selectivity. Further optimization has produced a series of compounds that are orally active, can penetrate the blood-brain barrier very effectively, and provide a sustained duration of action. This was illustrated with GT-2227, GT-2260, and GT-2286, which show similar CNS penetration profiles in ex vivo binding studies. These penetration profiles are better than those of clobenpropit, GT-2231, and thioperamide. Moreover, GT-2227 had an excellent time course profile with near maximal receptor occupancy maintained out to 8 h. This profile was notably better than that of thioperamide. Additional studies by Tedford et al. (1998b) have also characterized the HA H_3 functional antagonist activity of GT-2227 using the isolated guinea pig jejunum and cardiac synaptosomes. There, the pA_2 for GT-2227 was determined to be 7.9 for the inhibition of neurogenic contractions of the guinea pig jejunum, which is in agreement with the K_i for GT-2227.

Several studies have indicated a role for HA in cognitive processes (de Almeida and Izquierdo, 1988; Kamei and Tasaka, 1991; Kamei et al., 1993; Prast et al., 1996). These observations, combined with the neuroanatomical localization of HA H_3 receptors in cerebral cortical regions (Cumming et al., 1991; Pollard et al., 1993), are supportive of a functional role for HA in higher learning. Recently, the HA H_3 antagonists thioperamide and GT-2016 were shown to enhance learning and recall in several cognitive models (Meguro et al., 1995; Miyazaki et al., 1995; Tedford, 1998a). In addition, the in vivo and in vitro release of acetylcholine was shown to be modulated by presynaptic HA H_3 heteroreceptors (Clapham and Kilpatrick, 1992; Blandina et al., 1996). Blandina et al. (1996) further showed that in addition to reduced in vivo cerebral cortical acetylcholine release, the HA H_3 agonists imetit and (*R*)-α–methylhistamine impaired cognitive performance in both object recognition and PAR learning models. Together, these biochemical and behavioral studies are supportive of a role for HA and HA H_3 receptor blockade in cognition enhancement. The present studies show that GT-2227 produced significant improvements in the performance (latency) of developmentally immature rat pups in the PAR at doses which provide for 50 to 100% occupancy of cerebral cortical HA H_3 receptors in vivo. These doses of GT-2227 had no effect on the overall locomotor activities of adult rats as measured by total, horizontal, central and peripheral activities, stereotypes, and vertical movements.

Although the use of selective HA H_1 and H_2 ligands has been effectively exploited in the treatment of allergy and excessive gastric acid secretion, respectively, the pharmacological benefits of selective HA H_3 compounds have yet to be fully realized. Potential CNS clinical uses have been identified for both HA H_3 receptor antagonists or agonists based on the reports of the high density of HA H_3 receptor levels and their distribution within the CNS combined with the role of the HA H_3 receptor at both auto- and heteroreceptor sites. A therapeutic role for the use of HA H_3 receptor antagonists in cognitive dysfunction, sleep disorders, obesity, and hormonal dysfunction has been suggested (Timmerman, 1990; Leurs et al., 1998, Tedford, 1998a). The limited availability of clinical agents, however, has hindered further exploration of the clinical usefulness of such agents. Furthermore, the biochemical and pharmacological characterization of the HA H_3 receptor has been limited as well due to the poor CNS availability of a variety of early HA H_3 agents. The present studies describe the development of a new series of potent and selective HA H_3 receptor ligands with excellent CNS penetration. Initial behavioral assessment of a prototype compound (GT-2227) indicates that improvements in learning are seen at doses that provide significant cerebral cortical HA H_3 receptor occupancy without locomotor stimulant properties. These studies lend support for the use of HA H_3 receptor antagonists in diseases associated with cognitive or attentional deficits. Importantly, these new CNS-penetrating ligands can be used to conduct behavioral assessments to further explore the full potential and clinical utility of blockade of the HA H_3 receptor.

Acknowledgments

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Neurochemical and Behavioral Effects of Ciproxifan, A Potent Histamine H_3-Receptor Antagonist

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ABSTRACT

Ciproxifan, *i.e.*, cyclopropyl-(4-(3-1*H*-imidazol-4-yl)propyloxy) phenyl) ketone, belongs to a novel chemical series of histamine H_3-receptor antagonists. *In vitro*, it behaved as a competitive antagonist at the H_3 autoreceptor controlling [^{3}H]histamine release from synaptosomes and displayed similar K_i values (0.5–1.9 nM) at the H_3 receptor controlling the electrically-induced contraction of guinea pig ileum or at the brain H_3 receptor labeled with [^{125}I]iodoproxyfan. Ciproxifan displayed at least 3-orders of magnitude lower potency at various aminergic receptors studied in functional or binding tests. *In vivo*, measurement of drug plasma levels, using a novel radioreceptor assay in mice receiving ciproxifan p.o. or i.v., led to an oral bioavailability ratio of 62%. Oral administration of ciproxifan to mice enhanced by ~100% histamine turnover rate and steady state level of *tele*-methylhistamine with an ED_{50} of 0.14 mg/kg. Ciproxifan reversed the H_3-receptor agonist induced enhancement of water consumption in rats with and ID_{50} of 0.09 ± 0.04 mg/kg, i.p. In cats, ciproxifan (0.15–2 mg/kg, p.o.) induced marked signs of neocortical electroencephalogram activation manifested by enhanced fast-rhythms density and an almost total waking state. In rats, ciproxifan enhanced attention as evaluated in the five-choice task performed using a short stimulus duration. Ciproxifan appears to be an orally bioavailable, extremely potent and selective H_3-receptor antagonist whose vigilance- and attention-promoting effects are promising for therapeutic applications in aging disorders.

HA is a cerebral neurotransmitter exerting its actions on target cells via three classes of molecularly and/or pharmacologically well defined receptors designated H_1, H_2 and H_3 (reviewed by Hill *et al.*, 1997; Schwartz *et al.*, 1991, 1995). The H_3 receptor is a presynaptic receptor regulating the synthesis and/or release of HA itself (Arrang *et al.*, 1983) as well as a variety of other aminergic or peptidergic neurotransmitters (reviewed by Schlicker *et al.*, 1994). It was initially defined by the design of two selective ligands: (R)α-MeHA, a full agonist, and thioperamide, an antagonist with nanomolar potency ($K_i \cong 4$ nM). Thioperamide has become the prototypical H_3-receptor antagonist, used in a large number of neurochemical, electrophysiological and behavioral studies because it is one of the few agents able to markedly enhance cerebral histaminergic transmissions *in vivo* via a selective mechanism. In agreement, few other actions of thioperamide were described, *e.g.*, inhibition of P450 cytochromes (La Bella *et al.*, 1992) and 5-HT_3-receptor blockade (Leurs *et al.*, 1995), which require higher drug concentrations than H_3-receptor blockade and are therefore not relevant for *in vivo* studies.

Nevertheless thioperamide has several drawbacks: 1) its *in vivo* potency is rather low compared with its *in vitro* potency, suggesting that drug bioavailability, particularly its brain penetration, is restricted, 2) more importantly it displays a distinct liver toxicity on repeated administration which has prevented it being submitted to human clinical trials.

Because H_3-receptor antagonists represent a novel class of agents with potentially interesting therapeutic applications, namely in psychiatry (Schwartz *et al.*, 1995) sustained efforts have been devoted to the design of drugs more potent and safer than thioperamide (reviewed by Stark *et al.*, 1996b).

As with thioperamide, all highly effective compounds obtained so far contain a monosubstituted imidazole ring, but

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ABBREVIATIONS: HA, histamine; t-MeHA, *tele*-methylhistamine; (*R*)α-MeHA, (*R*)α-methylhistamine; AUC, area under the curve; C_{max}, maximal concentration; W, wakefulness; S1, light slow wave sleep; S2, deep slow wave sleep; PS, paradoxical sleep; EEG, electroencephalogram.

the thiourea moiety of the latter, to which hepatotoxicity might be attributable, is replaced by numerous polar functionalities such as amine or carbamate, etc.

Recently, we have designed 3-(1*H*-imidazol-4-yl)propanol derivatives as a novel series of potent *in vitro* H_3-receptor antagonists with a high capacity for oral absorption and brain penetration in some compounds (Stark *et al.*, 1996a; Hüls *et al.*, 1996). In these novel chemical classes of compounds, we have recently identified [^{125}I]iodoproxyfan, *i.e.*, [^{125}I]3-(1*H*-imidazol-4-yl)propyl-(4-iodophenyl)methyl ether as a new probe for a sensitive assay and localization of the H_3 receptor in brain (Ligneau *et al.*, 1994).

We describe the biological properties of ciproxifan (fig. 1), a highly potent and selective H_3-receptor antagonist belonging to this novel chemical class of compounds which suggest its potential therapeutic interest as a waking and procognitive agent.

Materials and Methods

[^{3}H]Histamine release from synaptosomes. [^{3}H]HA release experiments were performed according to Garbarg *et al.* (1992). Briefly, a crude synaptosomal preparation from rat cerebral cortex was preincubated for 30 min with [^{3}H]L-histidine (0.4 μM) at 37°C. After extensive washing synaptosomes were resuspended in fresh 2 mM K^+-Krebs-Ringer's medium and in the presence of the appropriate drugs. After 5-min incubation synaptosomes were depolarized bringing the K^+-concentration to 30 mM for 2 min. Incubations were ended by a rapid centrifugation and [^{3}H]HA levels in the supernatant were determined after an ion-exchange chromatography purification. Release was expressed as the percent fraction of total [^{3}H]HA initially present in the synaptosomal preparation. Typically total [^{3}H]HA represented about 3,500 dpm/mg protein and total radioactivity about 100,000 dpm/mg protein in the test tube.

Assay of t-MeHA in brain. Male Swiss mice (18–20 g) or male Wistar rats (140–160 g) (Iffa-Credo, L'Arbresle, France) were fasted for 24 hr before p.o. administration. After treatment animals were killed by decapitation, the brain was dissected out and homogenized in 10 volumes (w/v) of ice-cold perchloric acid (0.4 N). The clear supernatant obtained after centrifugation (2000 × *g*, 30 min, + 4°C) was stored at −20°C before measuring the t-MeHA level by radioimmunoassay as described (Garbarg *et al.*, 1992). Changes were evaluated statistically by the Student's *t* test.

[^{125}I]Iodoproxyfan binding assays. The procedure for binding assays to rat striatal brain membranes was that described by Ligneau *et al.* (1994). Aliquots of membrane suspension [100 μl containing 15 to 20 μg of protein determined according to Lowry *et al.* (1951) using bovine serum albumin as standard] were incubated for 60 min at 25°C with 25 pM [^{125}I]iodoproxyfan (K_d = 65 ± 4 pM) alone or together with competing drugs dissolved to give a final volume of 200 μl in a phosphate buffer medium (Na_2HPO_4/KH_2PO_4 50 mM, pH 6.8). Incubations performed in triplicate were stopped by four additions of 5 ml ice-cold medium followed by rapid filtration through glass microfiber filters (GF/B, Whatman, Maidstone, U.K.) presoaked in a 0.3% polyethylene imine ice-cold buffer. Radioactivity trapped on filters was measured on a gamma counter.

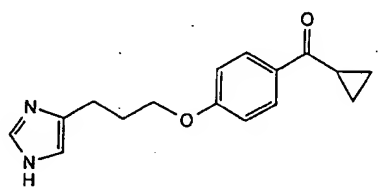

Fig. 1. Chemical structure of ciproxifan, *i.e.*, cyclopropyl-(4-(3-(1*H*-imidazol-4-yl)propyloxy)phenyl) ketone.

Histamine H_1 receptor assay on guinea pig ileum. The procedure used was that described by Pertz and Elz (1995).

Histamine H_2 receptor assay on guinea pig right atrium. The procedure used was that described by Pertz and Elz (1995).

Muscarinic M_3 receptor assay on guinea pig ileum. The procedure used was that described by Pertz and Elz (1995).

Adrenergic α_{1D} receptor assay on rat aorta. The procedure used was that described by Hirschfeld *et al.* (1992).

Adrenergic β_1 receptor assay on guinea pig right atrium. The procedure used was that described by Pertz and Elz (1995).

Serotoninergic 5-HT_{1B} receptor assay on guinea pig iliac artery. The procedure used was that described by Pertz (1993).

Serotoninergic 5-HT_{2A} receptor assay on rat tail artery. The procedure used was that described by Pertz and Elz (1995).

Serotoninergic 5-HT_3 receptor assay on guinea pig ileum. The procedure used was that described by Elz and Keller (1995).

Serotoninergic 5-HT_4 receptor assay on rat esophagus. The procedure used was that described by Elz and Keller (1995).

Histamine H_3 receptor assay on guinea pig ileum. The procedure used was that described by Ligneau *et al.* (1994). Briefly, longitudinal muscle strips from guinea pig small intestine were dissected out and incubated in a gassed O_2/CO_2 (95%/5%) modified Krebs-Ringer's bicarbonate medium at +37°C in presence of 1 μM mepyramine to block the H_1 receptor. After equilibration, contractile activity under stimulation (rectangular pulses of 15 V, 0.5 msec, 0.1 Hz) was recorded. Concentration-response curves of the effect of (*R*)α-MeHA alone or together with the antagonist were established.

Radioreceptor assay of H_3-receptor ligands in serum. Male Swiss mice (18–20 g, Iffa-Credo, L'Arbresle, France) were fasted for 24 hr before ciproxifan administration. At various times thereafter, mice were decapitated. Blood was collected at +4°C, serum collected after centrifugation (100 × *g*, 10 min, +4°C), and stored at −20°C. Ciproxifan levels were measured with the following radioreceptor assay derived from the [^{3}H](*R*)α-MeHA binding assay (Garbarg *et al.*, 1992).

Male Wistar rats (160–180 g, Iffa-Credo, L'Arbresle, France) were decapitated and the brain was removed immediately. The cerebral cortex was dissected out and homogenized in 10 volumes (w/v) of ice-cold 50 mM Na_2HPO_4/KH_2PO_4 buffer, pH 7.5 using a Polytron. After a centrifugation (140 × *g*, 10 min, +4°C), the supernatant was recentrifuged (23,000 × *g*, 10 min, +4°C). The last pellet was washed superficially with and then resuspended in fresh ice-cold phosphate buffer to constitute the membrane fraction used for the binding assay. One-ml aliquots of the membrane suspension containing 300 to 330 μg of protein [determined according to Lowry *et al.* (1951) using bovine serum albumin as standard] were incubated for 60 min at +25°C with 1 nM of [^{3}H](*R*)α-MeHA alone or together with different concentrations of ciproxifan in diluted serum of ciproxifan-free mice (standardization curve) or with diluted serum samples of ciproxifan-treated mice. Specific binding was defined as that inhibited by 3 μM thioperamide. Incubations were performed in triplicate and stopped by four additions of 5 ml of ice-cold phosphate buffer followed by rapid filtration through glass microfiber filters (GF/C, Whatman, Maidstone, U.K.) presoaked in 0.3% polyethylene imine ice-cold phosphate buffer. Radioactivity trapped on filters was measured by liquid scintillation spectrometry (Wallac 1410, EG&G, Evry, France). The standardization curves were established using a one-site competition model (GraphPad Prism, San Diego, CA) and H_3-receptor ligand concentrations in serum were calculated using these curves and expressed as ciproxifan concentrations. In the conditions retained, the detection limit of the radioreceptor assay corresponded to a concentration of 20 nM ciproxifan in serum. Changes in H_3-receptor ligand concentrations in serum with time were analyzed using either a point to point nonlinear model or the two phase exponential decay analysis model (GraphPad Prism).

(*R*)α-MeHA-induced water consumption in rats. The procedure used was that described by Clapham and Kilpatrick (1993) with slight modifications. Briefly, male Lister hooded rats (280–320 g,

Charles River, St Aubin lès Elbeuf, France), housed in cages of 10, were allowed free access to food and water. Experiments were carried out between 10 A.M. and 1 P.M. Drugs in 0.9% NaCl solution were administered i.p., each rat receiving two injections [vehicle or (*R*)α-MeHA 10 mg/kg/p.o., and vehicle or ciproxifan] of 250 μl each. After drug treatments, rats were returned to their home cage with food (standard pellet) and without water for 30 min. Then they were placed in individual cages with only a water supply and 10 min later, the amount of water consumed was recorded by weighing. Statistical evaluation of results was performed by Student's *t* test.

Analysis of neocortical EEG power spectral density and sleep-wake in the cat. Cats, a species in which the role of the H_3 receptor in sleep-wake control has been demonstrated, were used in this experiment (Lin *et al.*, 1990). Briefly, five adult cats of both sexes weighing 2.7 to 3.8 kg were chronically implanted, under pentobarbital anesthesia (25 mg/kg, i.v.), with electrodes for polygraphic recordings of neocortical and hippocampal EEG, ponto-geniculo-occipital activity, electromyogram and electrooculogram. In addition, a thermistor (10 K3 MCD2, Betatherm, 10 KΩ at 25°C, outer diameter of 0.45 mm) was placed in the caudate nucleus to record brain temperature. After a recovery period of 7 days the cats were housed in a sound-attenuated and dimly illuminated cage set at 24 to 26°C and fed daily at 6 P.M. with a normal standard diet. Polygraphic recordings were performed for 4 days to obtain the basic qualitative and quantitative parameters of the sleep-wake cycle.

Ciproxifan at doses of 0 (placebo), 0.15, 0.3, 0.7 and 2 mg/kg was administrated orally at 11 A.M. Subsequent polygraphic recordings were made for at least 24 hr. They were then scored minute by minute according to previously described criteria (Lin *et al.*, 1990) for wakefulness (W), light slow wave sleep (S1), deep slow wave sleep (S2) and paradoxical sleep (PS). In some animals, neocortical EEG signals from the first 6 hr after placebo or drug administration were digitized at a sample rate of 128 Hz and computed on a CED 1401 Plus (Cambridge Electronic Design, Cambridge, U.K.). The power spectral density was averaged over 30-sec epochs for the frequency range of 0.25 to 50 Hz by a Fast Fourier Transform routine using the CED program Spike2 and correlated with sleep-wake stages.

Five-choice task in rats. Male Lister hooded rats (Olac, Bicester, U.K.) were housed in pairs in a temperature-controlled (21°C) room that was illuminated in accordance with an alternating 12-hr light/dark cycle. Rats were food deprived and maintained at 85% of their free-feeding weight (MRC Diet 41B laboratory food) throughout the experiment while water was available *ad libitum*.

The test apparatus and procedure were as described in detail by Muir *et al.* (1994). Rats were trained to discriminate a brief visual stimulus presented in one of five spatial location during 30-min sessions. Rats initiated a trial by opening the magazine panel. After a 5-sec inter-trial interval a light at the rear of one of the five apertures was illuminated for 0.5 sec. Correct responses to the stimulus location were rewarded with the delivery of food pellets. Having obtained stable performance on the task (>80% correct responses), attentional demand of the task was increased by reducing the stimulus duration to 0.25 sec in certain drug sessions as described previously (Muir *et al.*, 1994, 1995). Drugs or the vehicle (saline) were administered i.p. 1 hr before the test session (0.5- or 0.25-sec stimulus duration) according to a Latin square design. A rest day followed by a baseline day separated each drug test day.

Analysis of data. Maximal effects, ED_{50}, EC_{50} and IC_{50} values were determined using an iterative computer least-squares method derived from that of Parker and Waud (1971) with the following nonlinear regression: effect of the drug = ([maximal effect of the drug].[drug dose-or-concentration])/([drug dose-or-concentration] + (ED_{50} or EC_{50} or IC_{50})).

K_i values of H_3-receptor antagonists are calculated from their IC_{50} values, assuming a competitive antagonism and by using the relationship (Cheng and Prussoff, 1973):

$$K_i = IC_{50}/(1+(S/K_d))$$

where S and K_d represent respectively either the concentration and the dissociation constant of the radioligand in binding experiments, or the concentration of HA and its EC_{50} in [^{3}H]HA release experiments. When a fixed concentration of ciproxifan, tested as an antagonist, was added to increasing imetit concentrations, the K_i value of ciproxifan was calculated from the following equation (Cheng and Prussoff, 1973)

$$K_i = I/[(EC_{50}'/EC_{50})-1]$$

where EC_{50} and EC_{50}' are the imetit concentrations required to obtain half-maximal inhibition of release in the absence and presence of ciproxifan respectively, and I is the ciproxifan concentration.

Radiochemicals and drugs. [^{125}I]Iodoproxyfan and [^{3}H](*R*)α-MeHA (specific activities at reference date of 2000 and 38.0 Ci/mmol, respectively) were from Amersham (Amersham, U.K.). All drug doses are expressed as free base of compound. Administration to animals was performed with drug preparation in 1% methylcellulose for the oral route, in 0.9% NaCl for i.v. and i.p. routes. The drugs and their sources were as follows: ciproxifan [cyclopropyl-(4-(3-(1*H*-imidazol-4-yl)propyloxy)phenyl) ketone] synthesis was described in the patent application (Schwartz *et al.*, 1996); thioperamide and (*R*)α-MeHA (Laboratoire Bioprojet, Paris, France); carboperamide [1-(heptanoyl)-4-(1*H*-imidazol-4-yl)piperidine] was from M. Robba (University of Caen, France); clobenpropit was provided by H. Timmerman (Vrije Universiteit, Amsterdam, The Netherlands); iodoproxyfan was synthesized at the Freie Universität Berlin; imetit was synthesized by S. Athmani (University College, London, U.K.). All other chemicals were obtained from commercial sources and were of the highest purity available.

Results

Effects of ciproxifan on H_3-receptor functional models in vitro. The H_3-receptor agonist imetit inhibited the [^{3}H]HA release with a maximal effect of 54 ± 2%. Pharmacological parameters were estimated using the H_3-receptor controlled inhibition curve of the [^{3}H]HA release. Imetit concentration required for half inhibitory effect of its own maximal effect was 1.6 ± 0.3 nM and the corresponding pseudo-Hill coefficient (n_H) of this concentration response curve was close to unity (0.85 ± 0.15). Ciproxifan (15 nM) induced a parallel rightward shift of the concentration-response curve for imetit and tended by itself to increase (~20%) the K^+-induced [^{3}H]HA release; the antagonism developed by ciproxifan was entirely surmounted in the presence of the highest imetit concentrations tested (fig. 2). The half-maximal inhibitory concentration for imetit in the presence of ciproxifan (estimated considering the total H_3-receptor controlled inhibition curve) was 12.8 ± 1.8 nM, leading to an apparent K_i value of 1.9 ± 0.3 nM for the antagonist.

Histamine (1 μM) inhibited its own release by 55 ± 2%, and a series of H_3-receptor antagonists progressively reversed this response with n_H coefficients close to unity (fig. 3). IC_{50} values (nM) of the various compounds were 9.2 ± 1.8 (ciproxifan), 75 ± 19 (thioperamide), 377 ± 38 (carboperamide), 11 ± 3 (clobenpropit) and 99 ± 5 (iodoproxyfan) leading to K_i values reported in table 1, assuming a competitive inhibition.

Ciproxifan (3–300 nM) competitively antagonized the (*R*)α-MeHA induced relaxation of the electrically stimulated guinea pig ileum longitudinal muscle (Ligneau *et al.*, 1994) without significantly affecting the maximum response of the H_3-receptor agonist in the absence of ciproxifan [91 ± 5 (3 nM ciproxifan, n = 2), 100 ± 4 (10 nM, n = 6), 110 ± 10 (30 nM,

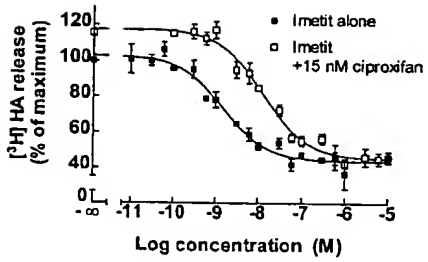

Fig. 2. Effect of ciproxifan on the inhibition by imetit of the K^+-induced [^{3}H]HA release from synaptosomes of rat cerebral cortex. Synaptosomes preincubated with [^{3}H]L-histidine were incubated for 5 min in the presence of imetit in increasing concentrations, alone or together with ciproxifan at a fixed (15 nM) concentration. They were subsequently depolarized for 2 min in the presence of 30 mM K^+ (final concentration). The spontaneous efflux of [^{3}H]HA (2 mM K^+) represented 14 ± 2% of the total [^{3}H]HA in synaptosomes. In the presence of added agents, [^{3}H]HA release induced by 30 mM K^+ (expressed in percent of total [^{3}H]HA over spontaneous efflux) represented 17 ± 2%. Each point represents the mean ± S.E.M. of results from three different experiments with quadruplicate determinations each.

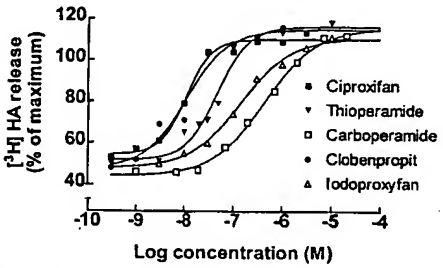

Fig. 3. Effects of H_3-receptor antagonists on the inhibition by HA of the K^+-induced [^{3}H]HA release from rat cerebral cortex synaptosomes. Synaptosomes preincubated with 1 μM HA alone or together with one of the compounds at increasing concentrations. They were depolarized for 2 min in the presence of 30 mM K^+. Each point represents the mean result from three different experiments with quadruplicate determinations each.

TABLE 1

In vitro and *in vivo* potencies of H_3-receptor antagonists

Compounds	*In Vitro* Tests (K_i, nM)		*In Vivo* Test (ED_{50}, mg/kg, p.o.)
	[^{3}H]HA release[a]	[^{125}I]Iodoproxyfan binding[b]	Increase in t-MeHA level in mouse brain[c]
Ciproxifan	0.5 ± 0.1	0.7 ± 0.2	0.14 ± 0.03
Thioperamide	4 ± 1	4 ± 1	1.0 ± 0.5
Carboperamide	20 ± 2	6 ± 2	3.9 ± 0.8
Clobenpropit	0.6 ± 0.2	0.5 ± 0.1	~25
Iodoproxyfan	5 ± 1	0.2 ± 0.1	>25

[a] Data from Fig. 3 except for thioperamide (from Arrang *et al.*, 1987) and iodoproxyfan (from Ligneau *et al.*, 1994).
[b] Data from Ligneau *et al.* (1994) except for ciproxifan (fig. 4).
[c] Data from figure 8 except for thioperamide (from Ganellin *et al.*, 1996) and iodoproxyfan (from Stark *et al.*, 1996).

n = 6), 105 ± 9 (100 nM, n = 6) and 87 ± 7% (300 nM, n = 6) *vs.* 100% relaxation in the absence of ciproxifan]. Schild analysis revealed a slope of 0.92 ± 0.04, not significantly different from unity (n = 26). After imposing the unity constraint, a pA_2 value of 8.38 ± 0.03 was calculated for ciproxifan.

Effect of ciproxifan and other H_3-receptor antagonists on [^{125}I]iodoproxyfan binding. At 25 pM [^{125}I]iodoproxyfan the specific binding to rat striatal membranes represented 8.4 ± 0.4 fmol/mg of protein, *i.e.*, 46 ± 1% of the total, and was completely and monophasically displaced by all compounds (fig. 4). From these displacement curves IC_{50}

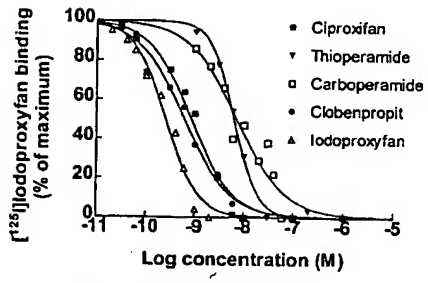

Fig. 4. Inhibition of [^{125}I]iodoproxyfan binding to rat striatal membranes by various histaminergic agents. Membranes were incubated for 60 min at 25°C with 25 pM [^{125}I]iodoproxyfan and unlabeled H_3-receptor antagonists in increasing concentrations. Specific binding, defined as that inhibited by 1 μM (*R*)α-MeHA, represented 46 ± 1% of the total binding. Results are expressed as percentages of specific [^{125}I]iodoproxyfan binding in the absence of unlabeled agents. Each point and vertical bars represent the mean ± S.E.M. of results from three different experiments with triplicate determinations each.

values for the compounds were deduced leading to the K_i values presented in table 1.

Receptor selectivity of ciproxifan. The compound displayed low apparent affinity at other receptor subtypes as evaluated in functional tests on isolated organs (histamine H_1 and H_2, muscarinic M_3, adrenergic α_{1D} and β_1, serotonin 5-HT_{1B}, 5-HT_{2A}, 5-HT_3 and 5-HT_4) (fig. 5). In addition, ciproxifan displayed a K_i value higher than 1 μM in a large variety of radioligand binding tests (Panlabs screen), except at [^{3}H]pirenzepine binding to rat cerebral cortex membranes where its K_i was about 1 μM (data not shown).

Changes in serum drug concentration in animals treated with ciproxifan. After i.v. administration of 1 mg/kg ciproxifan to mice (fig. 6B), the H_3-receptor ligand concentration in serum decreased progressively, fitting a typical biexponential decay model with half-times ($t_{1/2}$) of 13 and 87 min for the distribution and elimination phases, respectively. The quality of this fit is given by an R^2 value of 0.985. At 6 hr, the serum ligand concentration was still detectable with a value of 23 ± 6 nM. When ciproxifan was given orally, also at 1 mg/kg (fig. 5A), serum ligand level rose rapidly, being maximal at 30 min with a maximal concentration (C_{max}) value of 420 ± 40 nM; then, the ligand concentration

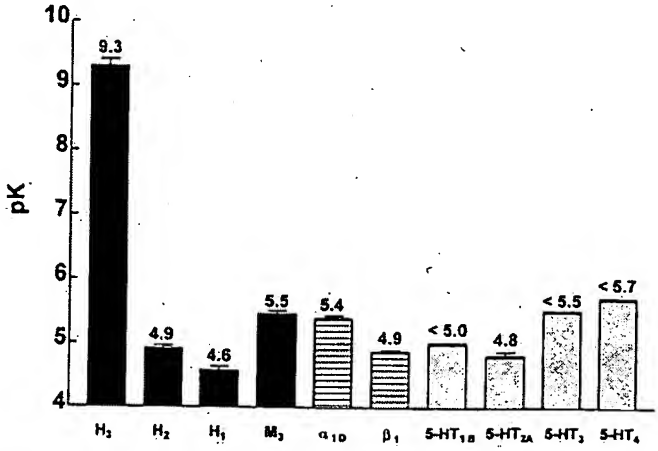

Fig. 5. Receptor selectivity profile of ciproxifan. The affinity of the compound at the H_3 receptor (pK_i from [^{3}H]HA release assay) is compared to corresponding values obtained in functional tests in isolated organs. Other values represent mean ± S.E.M. pA_2 (Arunlakshana and Schild, 1959) from n = 3 to 12 preparations except for the H_2- and β_1-receptor assay (pD'_2 according to Van Rossum, 1963).

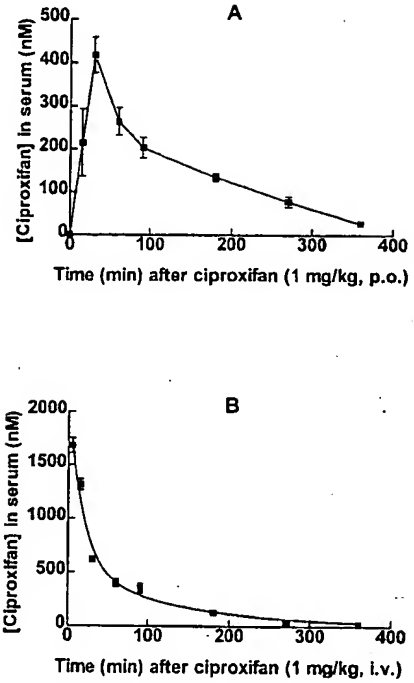

Fig. 6. Serum drug concentration in mice receiving ciproxifan. Mice were killed after p.o. (A) or i.v. (B) administration of ciproxifan (1 mg/kg), and drug concentrations were evaluated in serum by a radioreceptor assay. Means ± S.E.M. of values from six mice.

decreased but still remained measurable at 6 hr (27 ± 5 nM). The AUCs were 1425 and 890 nM.hr after i.v. and p.o. administrations, respectively, leading to an oral bioavailability coefficient (AUCp.o./AUCi.v..100%) of 62%.

In rats (n = 3) receiving 1 mg/kg ciproxifan p.o., the time-course of changes in serum ligand concentration were comparable to those in mice with a mean AUC of 2,225 nM.h, a C_{max} of 881 ± 322 nM, also observed at 30 min, and a level of 96 ± 4 nM at 6 hr (not shown).

In one cat receiving 3 mg/kg of ciproxifan orally serum levels (μM) were of 0.14 (0.5 hr), 4.0 (1 hr), 2.2 (1.5 hr), 0.27 (3 hr) and 0.12 (6 hr) leading to an AUC of 5.07 μM.hr at this dose.

Changes in brain t-MeHA level after administration of ciproxifan or other H_3-receptor antagonists. After the administration of ciproxifan (1 mg/kg, p.o.), brain t-MeHA level rose rapidly, being already significantly increased after 30 min, reaching a plateau between 90 and 180 min and still remaining enhanced after 270 min (fig. 7). In mice receiving pargyline, a monoamine oxidase inhibitor, t-MeHA level increased linearly with time at a rate of 55 ng/g/hr, whereas coadministration of pargyline and ciproxifan enhanced this rate to 120 ng/g/hr.

The dose-response curves of ciproxifan and a series of other H_3-receptor antagonists were established by measuring t-MeHA levels 90 min after oral administration (fig. 8). Ciproxifan, thioperamide and carboperamide, maximally increased t-MeHA level to an equivalent extents about 2-fold over basal values. However, clobenpropit, at the highest dosage tested (30 mg/kg, p.o.) maximally enhanced t-MeHA level by 45%, and iodoproxyfan (10 mg/kg, p.o.) did not significantly modify this level. The ED_{50} values of the compounds, derived from data of figure 8, are reported in table 1.

Similar experiments performed in rats receiving ciproxifan orally led to ED_{50} values (mg/kg) of 0.23 ± 0.04 in cerebral cortex, 0.28 ± 0.08 in striatum and 0.30 ± 0.08 in hypothalamus with similar maximal enhancements of about 100% (not shown).

The time-course of the changes in t-MeHA levels in mouse brain elicited by oral administration of 0.3 mg/kg ciproxifan and 3 mg/kg thioperamide (fig. 9) were analyzed in terms of AUCs, leading to values (in percent increase.hr) of 597 and 425, respectively.

Effect of ciproxifan on the water consumption induced by an H_3-receptor agonist. The H_3-receptor agonist (R)α-MeHA (10 mg/kg, i.p.) markedly enhanced water consumption in rats, an effect that was progressively reversed by coadministration of ciproxifan in increasing dosage, the ID_{50} of the antagonist being 0.09 ± 0.04 mg/kg (fig. 10). Ciproxifan alone (3 mg/kg) did not significantly modify water consumption (fig. 10).

Effect of ciproxifan in the five-choice task in rats. Analysis of variance revealed a significant drug × stimulus duration interaction [$F(1,9) = 12.19$, $P < .01$]. As shown in figure 11, reducing the duration of the visual stimulus to 0.25 sec resulted in a significant reduction in the accuracy of performance compared to the baseline (0.5 sec) stimulus condition. Newman Keuls *post hoc* comparisons revealed that this reduction in performance was significant and that choice accuracy significantly increased after administration of 3.0 mg/kg of ciproxifan under the shorter stimulus condition compared to performance after administration of the vehicle ($P < .05$). There was no significant effect of this manipulation of the stimulus duration or of ciproxifan on any of the other measures recorded (*i.e.*, speed, anticipatory or perseverative responses and errors of omission).

Effects of ciproxifan on neocortical EEG power spectral density and sleep-wake cycle in cat. Administration

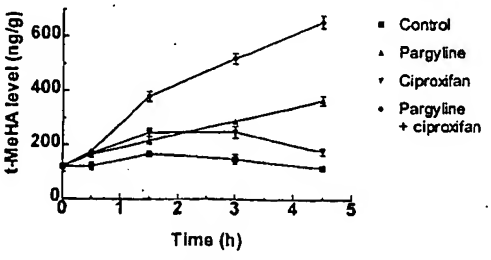

Fig. 7. Changes in brain t-MeHA levels in control and pargyline-treated mice after administration of ciproxifan. Mice were killed at various times after simultaneous administration of vehicle or ciproxifan (1 mg/kg, p.o.) and vehicle or pargyline (65 mg/kg, i.p.). t-MeHA levels are expressed in ng/g of tissue. Means ± S.E.M. of values from 12 mice.

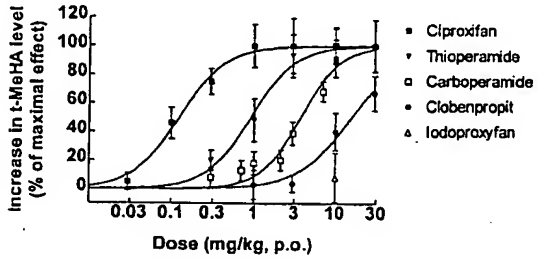

Fig. 8. Changes in brain t-MeHA levels in mice receiving H_3-receptor antagonists. Mice were killed 90 min after the p.o. administration of vehicle or drugs in increasing doses. t-MeHA levels in treated mice are expressed in percent increase as compared to levels in control mice (133 ± 7 ng/g). Means ± S.E.M. of values from 12 mice.

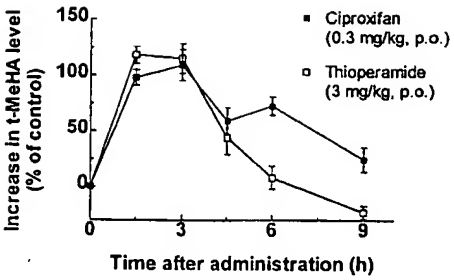

Fig. 9. Changes in brain t-MeHA levels in mice receiving ciproxifan or thioperamide. Mice were killed at various times after the p.o. administration of vehicle, ciproxifan (0.3 mg/kg) or thioperamide (3 mg/kg). t-MeHA levels are expressed in percent increase as compared to levels in corresponding controls. Means ± S.E.M. of values from 18 mice.

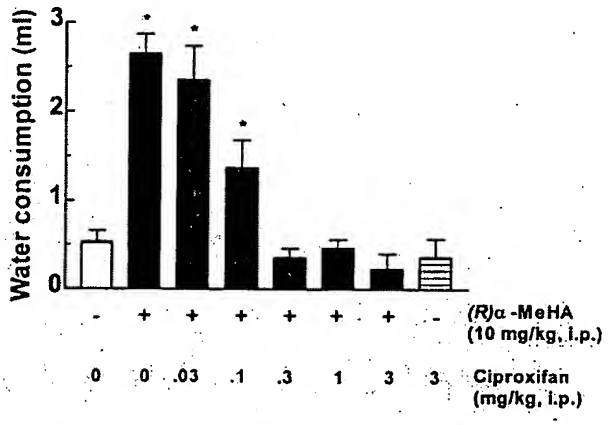

Fig. 10. Effect of ciproxifan on the (*R*)α-MeHA-induced water consumption in rats. Water consumption was measured over a 10-min period, 30 to 40 min after i.p. administrations of (*R*)α-MeHA and ciproxifan. Means ± S.E.M. of values from 5 to 10 rats per treatment group (data from two experiments). *P < .05 *vs.* vehicle.

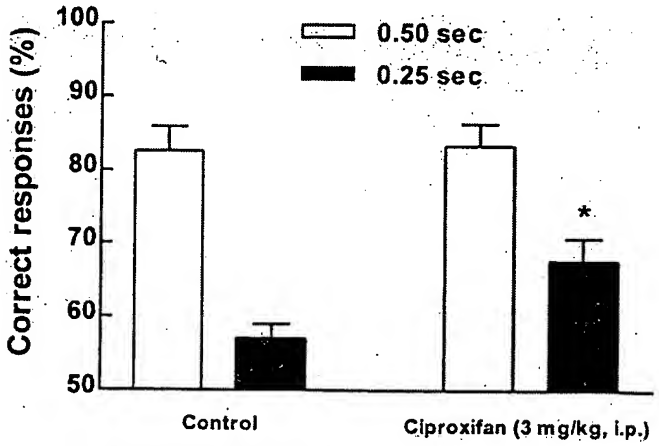

Fig. 11. Effects of ciproxifan in the five-choice task in rats. The drug was administered (3 mg/kg, i.p.) 1 hr before the test session. The accuracy of responding, expressed as the percentage of correct responses, was significantly (*P < .05) improved by treatment only when the stimulus duration was 0.25 sec instead of 0.50 sec.

of ciproxifan caused suppression or diminution (depending on the dose used) of neocortical slow activity (0.8–5 Hz) and spindles (8–15 Hz), resulting in a total cortical activation, *i.e.*, low voltage electrical activity with dominant waves in the β and γ bands (mainly 25–45 Hz). Furthermore, ciproxifan increased the power density of these neocortical fast rhythms (fig. 12). These effects, occurring within 25 min after

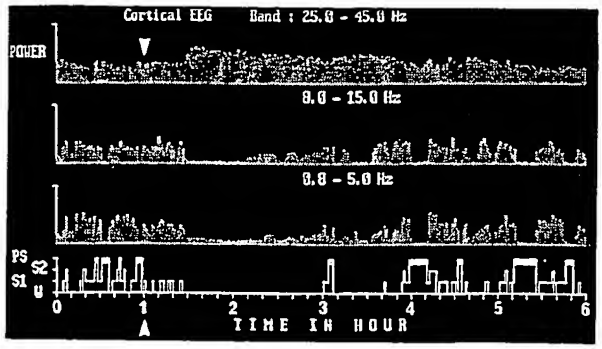

Fig. 12. An example of the effects of oral administration of ciproxifan on cortical EEG and sleep-wake cycle in cat. Neocortical EEG power density (mV^2) in different frequency bands and sleep-wake cycle 1 hr before and up to 5 hr after administration of 2 mg/kg ciproxifan (arrow) are given. Note, from bottom to top, that ciproxifan elicited a total waking state accompanied by a suppression of cortical slow frequency activity (0.8–5 Hz) and spindle (8–15 Hz) and a marked increase in fast rhythms (25–45 Hz). Abscissa, Time in hours; ordinate, sleep-wake stages (PS, paradoxical sleep; S2, deep slow wave sleep; S1, light slow wave sleep; W, wakefulness).

administration (fig. 12) were detectable at a dose of 0.15 mg/kg and became evident at a dose of 0.3 mg/kg or more (fig. 13).

The effects of ciproxifan on neocortical EEG were manifested on polygraphic scoring as an almost total waking state, the duration of which was dose dependent. This waking effect was characterized by an increase in wake-episode duration and a delayed sleep latency. During the same period both slow wave sleep (especially S2) and PS were suppressed (fig. 13). After the period of induced total waking, cortical slow activity gradually reappeared, but an increase in waking could be seen during a period proportional to the doses used. No obvious sleep rebound was noted after the waking effect, and all sleep-wake parameters including power spectral density of neocortical EEG returned to control levels on the next day. The arousal effects of ciproxifan (0.3 mg/kg) were prevented or significantly reduced by prior (15 min) systemic injection of either mepyramine (1 mg/kg), an H_1-receptor antagonist, or imetit (3 mg/kg), an H_3-receptor agonist (not shown).

Discussion

From our present *in vitro* and *in vivo* studies, ciproxifan appears as a pure competitive antagonist at the histamine H_3 receptor, one of the most potent so far available.

Our selection of the molecule was initially based on its ability to block the actions of histamine or imetit, a selective H_3-receptor agonist, at the autoreceptor regulating the release of neosynthesized [^{3}H]HA from K^+-depolarized synaptosomes, according to a previously described model (Arrang *et al.*, 1983; Garbarg *et al.*, 1989, 1992). On this model, ciproxifan induced a parallel rightward shift of the concentration-response curve to imetit, indicative of a competitive antagonism with an apparent dissociation constant in the low nanomolar range. Ciproxifan in increasing concentration also progressively blocked the (*R*)α-MeHA-induced relaxation of the electrically stimulated longitudinal muscle of guinea pig ileum in a competitive fashion. At these various functional models as well as the [^{125}I]iodoproxyfan binding tests, the drug displayed similarly low apparent dissociation constants

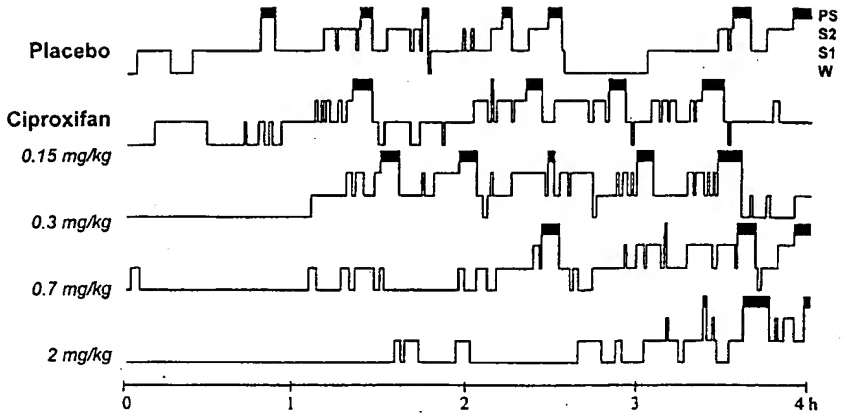

Fig. 13. Representative hypnograms (4 hr) obtained in cats after oral administration of ciproxifan at different doses. Note the dose-dependent waking effect. Abscissa, Time in hours; ordinate, sleep-wake stages (PS, paradoxical sleep; S2, deep slow wave sleep; S1, light slow wave sleep; W, wakefulness).

(K_i = 0.5–4.2 nM), an observation that does not support the hypothesis of the existence of H_3-receptor subtypes (Clapham and Kilpatrick, 1992). The hypothesis, mainly based on discrepancies in potency of some compounds in binding and functional models, is not supported either by the closely similar potencies of other antagonists in different models, examplified in table 1 (with the exception of iodoproxyfan for which the discrepancy can be fully explained by a slow equilibration rate).

The high degree of selectivity of ciproxifan toward the histamine H_3 receptor was shown by the observation that the drug displayed lower affinity by about three orders of magnitude for any other receptor subtype on which it was tested (see "Results").

Although some compounds *in vitro* were as potent as or even more potent than ciproxifan at the H_3 receptor, *e.g.*, clobenpropit and iodoproxyfan, ciproxifan given orally to mice enhanced brain t-MeHA levels at much lower dosage (ED_{50} = 0.14 mg/kg) than any of these compounds. A similar change, occurring with ED_{50} of 0.2–0.3 mg/kg p.o., was found in various areas of rat brain. t-MeHA is the product of the major metabolic pathway for endogenous HA in brain (Schwartz *et al.*, 1971), and its steady-state level is a reliable index of histaminergic neuron activity (Oishi *et al.*, 1983). The increase in t-MeHA level induced by the H_3-receptor antagonists corresponds to an enhanced HA release, reflecting the tonic inhibition that the endogenous amine exerts on this process and on neuronal firing via stimulation of autoreceptors in the somatodendritic or terminal area of histaminergic neurons. In agreement, ciproxifan (1 mg/kg, p.o.) enhanced HA turnover rate in mouse brain, evaluated from the rate of t-MeHA accumulation after monoamine oxidase inhibition, from a value of 55 ng/g/hr, consistent with corresponding values obtained in the same species using either isotopic (Verdière *et al.*, 1977) or nonisotopic methods (Oishi *et al.*, 1989), to a value of 120 ng/g/hr.

A similar maximal effect, corresponding to a nearly doubling of t-MeHA level, was obtained with thioperamide or carboperamide whereas, at the maximal dose tested of clobenpropit (30 mg/kg), this level was not reached and no significant change occurred after administration of iodoproxyfan (fig. 8) despite the high potency of these compounds *in vitro*.

In the case of ciproxifan, a rather high oral bioavailability was evidenced by the ratio (>60%) of AUCs of H_3 receptor binding activity in blood serum, measured by using a novel radioreceptor assay, following drug administration (1 mg/kg) by p.o. and i.v. routes, respectively. The rather slow kinetics of ciproxifan are indicated by a serum level still about 10 times above the K_i value of the drug at the H_3 receptor 6 hr after oral administration. At this time, t-MeHA levels in brain are still enhanced by 24 ± 11% (fig. 9). Such comparison between drug levels in blood and a typical brain response in rodents might be useful to predict effective dosages in other species, particularly humans, in which only blood levels are available, assuming a similar ability of the drug to cross the blood-brain barrier. A similarly favorable bioavailability of ciproxifan on oral administration to rats and cats is suggested by measurements of t-MeHA and drug serum levels, respectively (see "Results").

Characteristic behavioral responses were found in rats and cats receiving ciproxifan in low dosage. In water-deprived rats, ciproxifan blocked the enhancement of drinking elicited by (*R*)α-MeHA, an H_3-receptor agonist (Clapham and Kilpatrick, 1993), with an ID_{50} value of ~0.1 mg/kg. The exact site (central or peripheral) and mechanism of action of H_3-receptor ligands in this test has not been clearly established. Thus, whereas the involvement of the renin-angiotensin system in HA-induced drinking was postulated by Kraly and Miller (1982), an AT_1 antagonist did not affect the (*R*)α-MeHA-induced drinking (Clapham and Kilpatrick, 1993). The observation that (*R*)α-MeHA-induced drinking is blocked at doses of ciproxifan (this study), thioperamide and particularly clobenpropit (Barnes *et al.*, 1993), close to those enhancing endogenous HA release in brain (table 1), suggests that H_3 receptors in brain rather than in periphery are involved. The observation that ciproxifan or thioperamide given alone do not affect drinking suggests that the effect of (*R*)α-MeHA is mediated by H_3 hetero- rather than autoreceptors. In agreement, H_3 receptors on noradrenergic, serotoninergic, cholinergic, dopaminergic or peptidergic neurons do not appear to be, as with those on histaminergic neurons, tonically modulated by endogenous HA, because they respond to agonists but not to antagonists given alone (Schwartz *et al.*, 1991, 1995; Schlicker *et al.*, 1994).

The marked dose-dependent waking effect of ciproxifan in cats is consistent with a large variety of experimental evidence showing that histaminergic neurons play a prominent role in cortical activation and arousal in cats and rats (reviewed by Lin *et al.*, 1996; Schwartz *et al.*, 1991, 1995). The arousing effects of H_3-receptor antagonists, characterized by an enhancement of wakefulness at the expense of slow wave

and paradoxical sleep, was previously shown in both animal species using thioperamide (Lin *et al.*, 1990; Monti *et al.*, 1991) and carboperamide (Monti *et al.*, 1996). The effect of ciproxifan was, as in the case of thioperamide (Lin *et al.*, 1990), prevented by administration of mepyramine, an H_1-receptor antagonist, suggesting that it resulted from a H_3-receptor mediated enhancement of endogenous HA release. The brain site(s) at which endogenous HA promote(s) cortical EEG desynchronization via activation of the H_1 receptor could be one of the brain areas to which ascending or descending histaminergic pathways project known to express the H_1 receptor and to control sleep/wakefulness states. These potential targets comprise cortical neurons receiving direct histaminergic projections from the tuberomammillary nucleus, preoptic anterior hypothalamic neurons, thalamic relay neurons and basal forebrain or mesopontine tegmentum neurons (Lin *et al.*, 1996).

Because the effect of ciproxifan in cats was to enhance fast cortical rhythms, known to occur during increased vigilance, and to cause a quiet waking state, a positive outcome in attentional tests could be anticipated. In confirmation the drug significantly enhanced choice accuracy in the five-choice serial reaction-time task when a visual stimulus of short duration (0.25 sec) was used. Such reduction of the stimulus duration increases the attentional load placed on the task, reduces choice accuracy and has been used to observe the effects of cholinergic agents on attentional function (Muir *et al.*, 1994, 1995). "Pro-cognitive" effects of the H_3-receptor antagonist thioperamide have been reported in other behavioural tasks, *e.g.*, step-through passive avoidance response in senescence-accelerated mice (Meguro *et al.*, 1995); elevated plus-maze performance in mice with scopolamine-induced learning deficits (Miyazaki *et al.*, 1995) and in a test of social memory in rats (Prast *et al.*, 1996). However, it has also been reported that thioperamide failed to improve scopolamine-induced attentional dysfunction in the same 5-choice task used in the present study (Kirkby *et al.*, 1996).

Taken together these various observations suggest that ciproxifan is a potent, orally active H_3-receptor antagonist and it seems of interest to assess its potential therapeutic applications, namely in aging or degenerative disorders in which vigilance, attention and memory are impaired.

Acknowledgments

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Short communication

Effect of thioperamide, a histamine H_3 receptor antagonist, on electrically induced convulsions in mice

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The effect of thioperamide, a histamine H_3 receptor antagonist, on electrically induced convulsions was studied in mice. Thioperamide significantly and dose dependently decreased the duration of each phase of convulsion and raised the electroconvulsive threshold. Its anticonvulsant effects were prevented by pretreatment with (R)-α-methylhistamine, a histamine H_3 receptor agonist. These findings suggest that the effect of thioperamide on electrically induced convulsions is due to an increase in endogenous histamine release in the brain, an effect mediated by histamine H_3 receptors. The anticonvulsant effect of thioperamide was antagonized strongly by mepyramine (or pyrilamine), a centrally acting histamine H_1 receptor antagonist, but not by zolantidine, a centrally acting histamine H_2 receptor antagonist. Thus, the blockade by mepyramine of the thioperamide-induced decrease in seizure susceptibility indicates that histamine released by thioperamide from the histaminergic nerve terminals interacts with the histamine H_1 receptors of postsynaptic neurons. These findings support the hypothesis that the central histaminergic system is involved in the inhibition of seizures.

Histamine; Histamine H_3 receptor antagonists; Thioperamide; (R)-α-Methylhistamine; Convulsions (electrically induced)

1. Introduction

Histamine is a neurotransmitter or neuromodulator in the mammalian brain (Schwartz et al., 1991; Wada et al., 1991). Several lines of evidence have indicated that the central histaminergic neuron system plays an important role in the inhibition of convulsions (Tuomisto and Tacke, 1986; Yokoyama et al., 1992). Tuomisto and Tacke (1986) showed that metoprine, an inhibitor of histamine N-methyltransferase that increases brain histamine levels after its systemic administration, inhibited hindlimb extension after maximal electroshock in rats. In mice, the anticonvulsive effects of L-histidine, a precursor of histamine, on the duration of the clonic and convulsive phases of coma were found to depend on the brain level of histamine and to be mediated by central histamine H_1 receptors (Yokoyama et al., 1992). In addition, clinical reports have shown that histamine H_1 receptor antagonists occasionally induce convulsions in children, especially those of pre-school ages (Schwartz and Patterson, 1978).

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In addition to postsynaptic histamine H_1 and H_2 receptors, the existence of presynaptic histamine H_3 receptors that control the release of neuronal histamine has been suggested (Arrang et al., 1983). Thioperamide, a histamine H_3 receptor antagonist, enhances and (R)-α-methylhistamine, a histamine H_3 agonist, blocks histamine release from histaminergic presynapses (Arrang et al., 1987). Little is known about the effects of these drugs on various physiological parameters, because there are few reports on their effects on physiological functions in the central nervous system (Sakai et al., 1991; Scherkl et al., 1991).

In this study, we examined the effects of thioperamide and (R)-α-methylhistamine on electrically induced convulsions in mice. We used mepyramine and zolantidine (centrally acting histamine H_1 and H_2 receptor antagonists, respectively) to verify the involvement of histaminergic receptors on postsynaptic neurons in electrically induced convulsions.

2. Materials and methods

2.1. Animals

Six-week-old male ddY mice (Funabashi Farm Co., Funabashi, Japan) weighing 23–26 g were used. They

were housed in a room at 22 ± 2°C with a 12-h light/dark cycle (lights on at 7:00 h) and supplied with food and water ad libitum. All electroconvulsive shock experiments were performed between 13:00 and 16:00 h.

2.2. Electroconvulsive shock

Convulsions were induced as described earlier (Yokoyama et al., 1992). Briefly, electroconvulsive shock was induced by applying an electric current (100-Hz square waves of 30 mA for 0.1 s) through ear-clip electrodes attached with electroencephalograph paste. Seizure susceptibility was evaluated as the duration of the various phases of convulsion. The tonic phase was regarded as the period between the onset of hindlimb extension and the start of myoclonic jerks, the clonic phase as that during myoclonic jerks, and the convulsive coma phase as that between the end of myoclonic jerks and recovery of the righting reflex. Electroconvulsive thresholds were determined using the 'up-and-down method' described by Kimball et al. (1957).

2.3. Drugs

Thioperamide maleate (a gift from Sumitomo Pharmaceutical Co., Osaka, Japan) was suspended in saline containing 0.025% Tween 80 (Wako Pure Chem., Tokyo, Japan). Mepyramine maleate (Sigma Chem. Co. St. Louis, MO, USA) and zolantidine dimaleate (Smith-Kline Beecham Lab., London, UK) were dissolved in saline. These drugs were administered intraperitoneally (i.p.) in a volume of 0.1 ml/kg body weight. (R)-α-Methylhistamine dihydrochloride (a gift from Sumitomo Pharmaceutical Co., Osaka, Japan) was dissolved in Ringer's solution (Otsuka Pharmaceuticals, Tokyo, Japan) and administered intracerebroventricularly (i.c.v.), as described previously (Onodera and Shinoda, 1991). Doses of drugs are expressed in terms of the free bases.

2.4. Protocol

Thioperamide (3.75, 7.5 or 15 mg/kg) was administered i.p. 60 min before electroconvulsive shock. (R)-

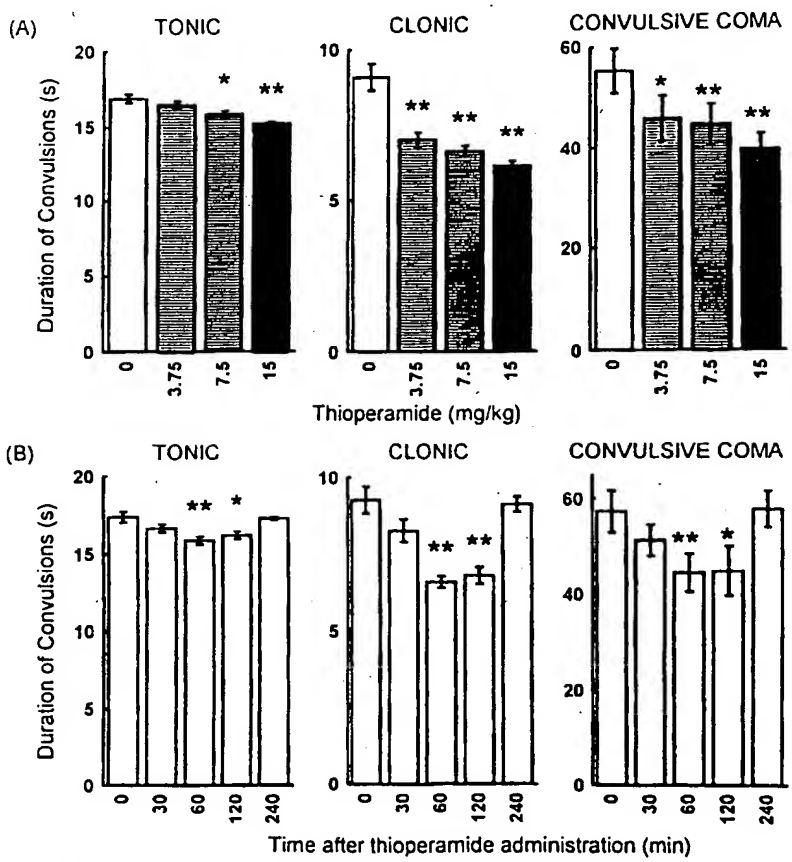

Fig. 1. (A) Effects of thioperamide on the duration of tonic, clonic and convulsive coma phases of electrically induced convulsions in mice (n = 10). Mice were subjected to electroshock 60 min after the i.p. administration of thioperamide (0, 3.75, 7.5 or 15 mg/kg). (B) Time course of the effects of thioperamide on electrically induced convulsions in mice (n = 10). Mice were subjected to electroshock 30, 60, 120 and 240 min after i.p. administration of thioperamide (7.5 mg/kg). The duration of convulsions was measured as described in the text. * $P < 0.05$ and ** $P < 0.01$ vs. the control group, significances of difference between groups by ANOVA followed by Duncan's test.

α-Methylhistamine (100 ng, 1 μg or 10 μg) was administered i.c.v. 15 min before electroconvulsive shock. Control mice received the same volume of saline containing 0.025% Tween 80 (i.p.) or Ringer solution (i.c.v.). In the time course studies, mice were treated with 7.5 mg/kg of thioperamide and were subjected to electroshock 0, 30, 60, 120 or 240 min later; the duration of the phases of convulsion were measured. Other mice were treated with thioperamide (7.5 mg/kg) or vehicle and were subjected to electroshock 60 min later. Mepyramine (3.7 mg/kg) or zolantidine (6.1 mg/kg) was administered i.p. 30 min before thioperamide. (R)-α-Methylhistamine (1 μg or 10 μg) or Ringer solution was administered i.c.v. 15 min before thioperamide.

2.5. *Statistical analysis*

The statistical significances of differences in the duration of the different phases of convulsion were determined by an analysis of variance (ANOVA) followed by Duncan's test. The statistical significances of differences in electroconvulsive thresholds were determined by using Litchfield and Wilcoxon's method.

3. Results

Figure 1A shows the effects of thioperamide on electrically induced convulsions in mice. The duration of the tonic phase was significantly decreased by i.p. administration of thioperamide at 7.5 or 15 mg/kg ($P < 0.01$). The decrease was dose dependent. The duration of the clonic and the convulsive coma phases was also significantly and dose dependently decreased by thioperamide. In contrast, (R)-α-methylhistamine (100 ng, 1 μg or 10 μg i.c.v.) did not affect the duration of any phase of convulsion (data not shown). Figure 1B shows the time course of the effects of thioperamide on electrically induced convulsions in mice. The maximum effect of thioperamide on the duration of each phase of convulsion was observed 60 min after its administration. The duration of the different phases of convulsion 30 and 240 min after the administration of

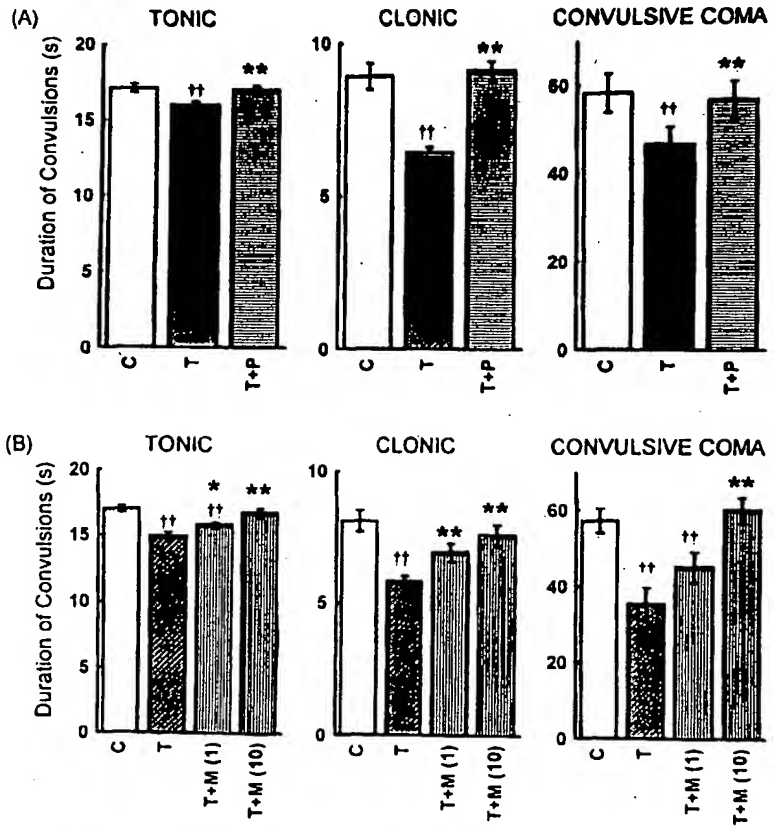

Fig. 2. Effect of thioperamide on electrically induced convulsions in mice (n = 10) and its antagonism by mepyramine (A) and (R)-α-methylhistamine (B). Mice were subjected to electroshock 60 min after i.p. administration of thioperamide (7.5 mg/kg). Mepyramine or (R)-α-methylhistamine treatment was as described in the text. Values are for treatment with vehicle, C; thioperamide, T; thioperamide and pyrilamine (mepyramine), T + P; thioperamide and (R)-α-methylhistamine (1 μg or 10 μg, respectively), T + M (1 or 10). Significant differences: * $P < 0.05$ and ** $P < 0.01$ vs. the thioperamide-treated group, † $P < 0.05$ and †† $P < 0.01$ vs. the control group, as determined by ANOVA followed by Duncan's test.

thioperamide was not significantly different from that of the control group.

Figure 2A shows the effects of thioperamide on electrically induced convulsions in mice and the antagonisms of these effects by mepyramine. The thioperamide-induced decrease in the duration of each phase of convulsion was fully antagonized by mepyramine (fig. 2A), but not by zolantidine (data not shown). Administration of mepyramine or zolantidine alone did not affect the duration of any convulsive phase (data not shown). Figure 2B shows the effects of thioperamide on electrically induced convulsions in mice and the antagonism of these effects by (R)-α-methylhistamine. The thioperamide-induced decrease in the duration of each phase of convulsion was fully antagonized by (R)-α-methylhistamine (10 μg).

The electroconvulsive threshold 60 min after the treatment with thioperamide at the doses of 0, 3.75, 7.5 and 15 mg/kg was 13.5 (11.9–15.3, 95% confidential limits, n = 15), 15.8 (14.2–17.6, $P < 0.05$), 19.7 (18.4–21.0, $P < 0.01$) and 20.8 (19.3–22.4, $P < 0.01$) mA, respectively. Thioperamide significantly and dose dependently raised the electroconvulsive threshold. The electroconvulsive threshold in the groups treated with vehicle, thioperamide with vehicle, and thioperamide with mepyramine was 13.1 (11.7–14.5), 20.2 (18.8–21.8, $P < 0.01$) and 14.0 (12.5–15.8) mA. Thus the effect of thioperamide on the electroconvulsive threshold was fully antagonized by mepyramine. However, zolantidine had no influence on the effects of thioperamide (data not shown). The electroconvulsive threshold in the groups treated with the vehicles, thioperamide with vehicle, and thioperamide with (R)-α-methylhistamine (10 μg) was 12.4 (10.1–15.2), 18.6 (16.6–21.0, $P < 0.01$) and 13.2 (10.7–16.2) mA, respectively. The effect of thioperamide on the electroconvulsive threshold was antagonized by (R)-α-methylhistamine. The electroconvulsive threshold in the groups treated with mepyramine, zolantidine or (R)-α-methylhistamine alone was not changed significantly (data not shown).

4. Discussion

Arrang et al. (1983) reported evidence for a local feedback mechanism regulating the release and synthesis of histamine through a pre-synaptic autoreceptor termed the histamine H_3 receptor, and also showed that thioperamide was a specific antagonist of histamine H_3 receptors (Arrang et al., 1987). Thioperamide induces a prolonged increase of histamine turnover, as indicated by changes in the content of histamine and N^{τ}-methylhistamine in the cortex of rats (Oishi et a., 1989). Mochizuki et al. (1991) observed an increase in endogenous histamine release in living rat brain after the administration of thioperamide, using microdialysis techniques. These authors suggested that presynaptic control mediated by histamine H_3 autoreceptors is a major mechanism for regulating the activity of histaminergic neurons.

The present results clearly showed that thioperamide decreased seizure susceptibility in mice with electrically induced convulsions. The duration of the different phases of convulsion and the electroshock threshold were used to evaluate seizure susceptibility. Both methods showed similar results; however, the measurement of the duration of the phases of convulsion was more reproducible with small numbers of animals.

The effects of thioperamide on seizure susceptibility were more pronounced at 60 min than at 30 or 240 min after its administration. The effects of thioperamide on locomotor activity in mice were also significant 60 min after its administration (Sakai et al., 1991). The anticonvulsant effect of thioperamide was prevented by pretreatment with (R)-α-methylhistamine, a histamine H_3 receptor agonist, although treatment with (R)-α-methylhistamine alone did not affect the seizure susceptibility of mice with electrically induced convulsions. These findings suggest that thioperamide acts by increasing endogenous histamine release through the histamine H_3 receptor system. The anticonvulsant effects of thioperamide were antagonized effectively by mepyramine, but not by zolantidine. Treatment with either mepyramine or zolantidine alone did not affect the duration of any phase of convulsion. Thus the blockade by mepyramine of the thioperamide-induced decrease in seizure susceptibility could be explained by an interaction of histamine released by thioperamide from histaminergic nerve terminals with histamine H_1 receptors on postsynaptic neurons.

In mice with electrically induced convulsions, (S)-α-fluoromethylhistidine, an inhibitor of histamine synthesis that decreases brain histamine levels, increased the duration of clonic convulsions, but not the duration of tonic convulsions, whereas L-histidine, a precursor of histamine, decreased the duration of clonic convulsions, but not the duration of tonic convulsions (Yokoyama et al., 1992). However, the present findings showed that thioperamide decreased the duration of both tonic and clonic convulsions. One possible explanation for the marked effects of thioperamide on tonic convulsions may be that neuronal histamine release modulates other neuron systems through histamine receptors (Philippu, 1991). The dose of thioperamide used in this study did not affect the brain levels of dopamine, 3,4-dihydroxypheniylacetic acid, serotonin, or 5-hydroxyindolacetic acid (Sakurai et al, personal communication). However, Schlicker et al. (1989) suggested that histamine H_3 receptors regulating the release of noradrenaline are located on catecholaminergic nerve endings. In vivo experiments demonstrated

that catalepsy induced by i.c.v. histamine can be evoked not only by histamine receptor activation but also by cholinergic and dopaminergic imbalance (Onodera and Shinoda, 1991). The increase in amphetamine- and apomorphine-induced locomotor activity was inhibited by thioperamide (Clapham and Kilpatrick, 1992). Furthermore, Kishino et al. (1989) suggested that neuronal histamine release is modulated by NMDA or GABA receptors.

In this study, we showed that thioperamide decreased the seizure susceptibility of mice with electrically induced convulsions. The anticonvulsive effect of thioperamide was antagonized by mepyramine or (R)-α-methylhistamine, indicating that histamine is released from histaminergic nerve terminals through histamine H_3 receptors and interacts with histamine H_1 receptors on postsynaptic neurons. These findings support the hypothesis that the central histaminergic neuron system is involved in the inhibition of seizures.

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Role of histamine H_3-receptors in the proliferation of neoplastic cells in vitro

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key words: proliferation of neoplastic cells, H_3 receptors

SUMMARY

The study was devoted to the investigation of the effect of tioperamide and clobenpropit — the antagonists, as well as R-alpha-methylhistamine and imetit — the agonists of histamine H_3 receptors on the proliferation of neoplastic cells in vitro. The study was performed upon the following cell lines: BM — human melanoma, A-431 — human cutaneous carcinoma, TT — human medullary thyroid carcinoma, Ta3Hauszka — mammary cancer of mice, F6BtY — normal bovine foetal thyroid. For each cell line, test value (TV) and test index (TI) were determined according to generally-used criteria. The study presented in our paper is the first attempt to use the modulation of histamine H_3 receptors in the regulation of neoplastic cells proliferation. It was demonstrated that antagonists of histamine H_3 receptors — tioperamide and clobenpropit considerably inhibit the proliferation of the cells of human neoplasms — melanoma and cutaneous carcinoma. An opposite effect was observed in the case of the agonists of these receptors — R-alpha-methylhistamine and imetit — which very clearly stimulate proliferation of these neoplastic cells. Non-neoplastic cells of normal bovine thyroid are slightly sensitive to the inhibiting activity of antagonists as well as to proliferation stimulating activity of the agonists of these receptors. The activity of the studied compounds may be associated with H_3 receptors, since the antagonists of these receptors inhibit proliferating action of agonists. These observations suggest that mediators released after the use of the antagonists of these receptors, or intracellular changes taking place as an effect of their action, slow down the proliferation of neoplastic cells, but not normal cells.

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INTRODUCTION

The studies carried out in recent years suggest that in the course of intracellular mechanisms, histamine may lead to the proliferation of cells and play some role in the growth of tissues and their reconstruction.

Great activity of histidine decarboxylase, an enzyme which catalyses the formation of histamine out of histidine, was found in rapidly developing tissues, e.g. in rat foetuses, healing wounds, some tumour cells and in skin tumour caused by the administration of phorbol esters [1,2,3,4]. It was also demonstrated that histamine intensifies proliferation of normal cells, such as alimentary tract epithelium or gastric carcinoma cells of Kato III line [5].

Histidine decarboxylase is widely spread and present in numerous cell types: mastocytes, skin cells, platelets, basophiles and carcinoid. As far as alimentary tract is concerned, high activity was detected in stomach — in mastocytes of mucosa and argentaffin cells, particularly in rats [6,7,8].

The growth of the number of mastocytes was demonstrated for instance in human uterine carcinoma and gastric carcinoma, as well as in transplantable sarcoma in rats. P 815 tumour was described in mice; it was made up exclusively of mast cells, which retained the ability of histamine and serotonin synthesis in vitro. Mast cell leukae-

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mias are also known in people and mice; IgE receptors are found on the surface of these cells. Since degranulation of mast cells releases heparin which has an anti-mitotic effect, it was suggested that mast cells play a role of proliferation-inhibiting cells. However, as leukaemia progresses, the number of mastocytes and eosinophiles declines.

On the other hand, it was found that the administration of monofluoromethylhistidine — an irreversible histidine decarboxylase inhibitor slows down the development of certain neoplasms [9].

The mechanism in which histamine leads to increased proliferation of cells is not clear.

As stimulation of presynaptic H_3 receptors with R-alpha-methylhistamine results in the inhibition of histamine synthesis and release through the inhibition of histidine decarboxylase [10,11,12,13], the assessment of the effect of agonists and antagonists of H_3 receptors upon the development of neoplastic cells may have some practical significance.

The aim of the study was an assessment of the effect of H_3 receptors agonists and antagonists upon the proliferation of neoplastic cells in vitro.

MATERIAL AND METHODS

Tests on cells cultured in vitro are based upon obtained proliferating cell cultures and their further development in the presence of studied compounds [14,15,16,17].

The study was performed upon cell lines of neoplasms cultured in the Laboratory of Cell Culture, of the Department of Histology and Embryology, Medical University in Wrocław.

These were cells lines of the following neoplasms:

BM — human melanoma
A-431 — human cutaneous carcinoma
TT — human medullary thyroid carcinoma
Ta3Hauszka — mice mammary carcinoma
and F6 BIY — normal bovine foetal thyroid.

All the cell lines grew in a monolayer manner, stuck to the bottom of culture container (Falcon bottles) filled with MEM fluid (Minimal Essential Medium of Eagle's manufactured by Gibco), with an addition of 10% FCS (foetal calf serum), 2 mM glutamine and 100 $\mu g/cm^3$ mixture of antibiotics (penicillin + streptomycin). The culture was placed in a special incubator at 37°C in 5% CO_2 and 95% oxygen. Trypsin and EDTA were used in order to remove cell lines from falcon bottles for the test.

TT line was conducted on L-15 culture fluid, which maintains stable pH and does not require CO_2 as a stabiliser, and therefore the culture was possible in hermetic bottles. The remaining ingredients were the same and in the same amounts as in MEM fluid.

All cell lines, including TT were placed on MEM culture fluid for the tests.

Preparations were dissolved in distilled water (Acqua pro iniectione) and subsequently filtered through a milli-leak filter, after which they were used in the investigations.

The experiment was divided into three stages:

1. The effect of tioperamide and R-alpha-methylhistamine on the proliferation of 5 cell lines.
2. The effect of various histamine receptor modulators upon the proliferation of selected 3 cell lines.
3. The effect of the antagonists of H_3 receptors upon the proliferation of neoplastic cells stimulated with agonists of these receptors.

For each cell line investigated in the first stage of the experiment, the following concentrations were used:

10^1 — 230 $\mu g/cm^3$
10^0 — 23 $\mu g/cm^3$
10^{-1} — 2.3 $\mu g/cm^3$
10^{-2} — 0.23 $\mu g/cm^3$

Additionally, preliminary tests were performed on TT cell lines — human medullary thyroid carcinoma with the use of the following concentrations of both studied preparations: 1, 5, 10, 50 and 100 $\mu g/cm^3$.

The results of these investigations as well as values of concentrations calculated theoretically in the way given below were useful for the determination of concentration values used in further investigations.

Since other studies indicate that tioperamide and R-alpha-methylhistamine are active e.g. in the doses of 15 mg/kg i. p, initial concentration of 23.1 $\mu g/cm^3$ were used for the investigations of oncostatic activity.

Test for each studied cell line — an outline of the experiment:

1. Establishing the culture
2. Preparing suspension of cells of 2 x $10^4/cm^3$ density — initial control

3. Counting the cells after 72 hours- preliminary control
4. Exchange of medium (addition of study preparations) for another 72 hours
5. Counting the cells — final results

Grown cells in the stage of active growth were removed from the bottle with trypsin and EDTA, after which they were counted and the suspension of 2 x $10^4/cm^3$ density was prepared. 1 cm^3 of the suspension was poured into each whole. Three days later, the fluid was replaced with a 'new' culture medium, and at the same time, the cells were counted in order to establish the growth of cells from the time of setting up the culture till the day of starting the test proper. This number was a preliminary control (Cp). Then, 1 cm^3 preparations in studied concentration were added to the new medium for 72 hours, and the cells were counted again, while the result obtained was a final control or — if the study preparation was added — a final result.

Test value (TV) and test index (TI) were calculated according to the formulae:

$$WT = \frac{Cx - Cp}{Cf - Cp} \times 100\% \qquad IT = WT \times \frac{Cp}{Cf}$$

Cx — number of cells after 72-hour incubation with study preparation

Cp — number of cells in preliminary control 72 hours after setting the plate

CF — number of cells in final control after another 72 hours

Ci — initial control — of identical number of cells per 1 whole (mean of 3) / 1 cm^3 of culture medium

Ci was 2 x 10^4 in all the investigations performed.

If the value of test index TI is lower than –81%, this signifies that the cells are sensitive to study compound. With values ranging between –40% and –80%, the cells are regarded slightly sensitive to study compounds, while with values exceeding –40%, it is assumed that the cells are not sensitive to study compounds. It should be pointed out that presented test values concern cytostatic drugs, which are considered standard preparations, with very strong activity [14,15,16,17].

RESULTS

Very strict criteria used for cytostatic drugs were accepted for the evaluation of the effect of study compounds on cell proliferation. Therefore, values lower than –80% are the evidence for a very strong proliferation inhibiting activity, which is characteristic for oncostatic agents.

The cells of human melanoma and cutaneous carcinoma proved sensitive to tioperamide in the concentration of 230 $\mu g/cm^3$. TI values are lower than –80% and equal –167% and –92.7%, respectively (table 1, figure 1).

The cells of human medullary thyroid carcinoma and mammary carcinoma of mice were slightly sensitive (TI ranging between –40% and –80%) to tioperamide in the same concentration, while the values of proliferation test — TI index — are –54% and –46%, respectively (table 1, figure 1)

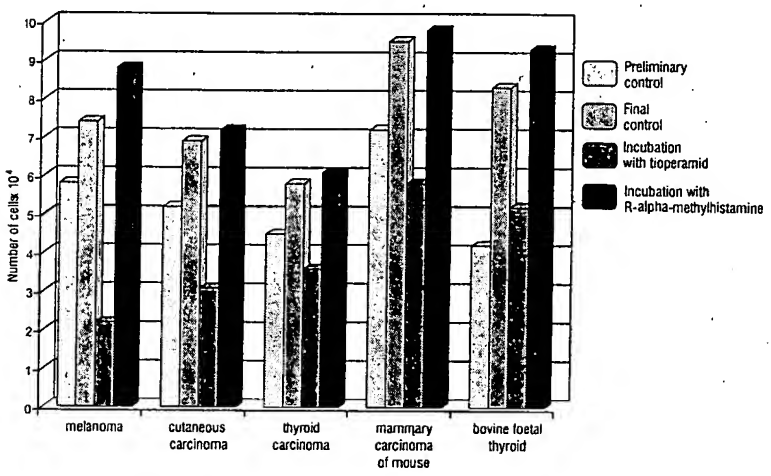

Figure 1. Effect of tioperamide and R-alpha-methylhistamine in the concentration of 230 $\mu g/cm^3$ each on cell proliferation.

Table 1. Effect of tioperamide and R-α-methylhistamine in the concentration of 230 μg/cm³ each, upon proliferation of studied cell lines

Neoplasm	Value	Tioperamide	R-α-methylhistamine
BM	WT	–213**%	188%
	IT	–167**%	147%
A–431	WT	–124**%	118%
	IT	–92.7**	89%
TT	WT	–69*%	123%
	IT	–54*%	95%
Ta3Hauszka	WT	–61*%	113%
	IT	–46*%	86%
F6BIY	WT	24%	124%
	IT	12%	63%

The cells of normal bovine foetal thyroid were not sensitive to tioperamide (TI = 12%) (table 1).

R-alpha-methylhistamine in the concentration of 230 μg/cm³ acts in an opposite way. Test values (TV) are clearly higher when compared to the corresponding values in the case of tioperamide, and they are 147% for human melanoma cells, 89% for human cutaneous carcinoma, 95% for human thyroid carcinoma, 86% for mammary carcinoma of mice and 63% for the cells of normal bovine foetal thyroid (table 1).

Antagonists of histamine H_3 receptor in lower concentrations also inhibited the development of human melanoma cells. Proliferation test indices for both antagonists of H_3 receptors are negative

Table 2. Effect of histamine receptor modulators upon proliferation of human melanoma cells.

Compound	Concentration μg/cm³	WT	IT
		%	
Clobenpropit	200	–66.7	–39.2
	100	–24.2	–14.2
Tioperamide	200	–60.6	–35.6
	100	–42.4	–24.9
R-α-methylhistamine	200	136.4	80.1
	100	115.2	67.7
Imetit	200	93.9	55.2
	100	106.1	62.3
Cimetidine	200	97.0	57.0
	100	118.2	69.4
Famotidine	200	90.9	53.4
	100	109.1	64.1

and they equal –39.2 and –14.2 for 200 μg/cm³ and 100 μg/cm³ clobenpropit, respectively, as well as –35.6 and –24.9 for tioperamide (table 2). However, this activity is weaker when compared with their activity in the concentration of 230 μg/cm³ (table 1).

An opposite direction of action was observed after the administration of agonists of histamine receptors R-alpha-methylhistamine and imetit as well as antagonists of histamine H2 receptors cimetidine and famotidine. All the four compound listed above increased the proliferation of human melanoma cells with the strength adequate to their concentration (table 2, figure 2).

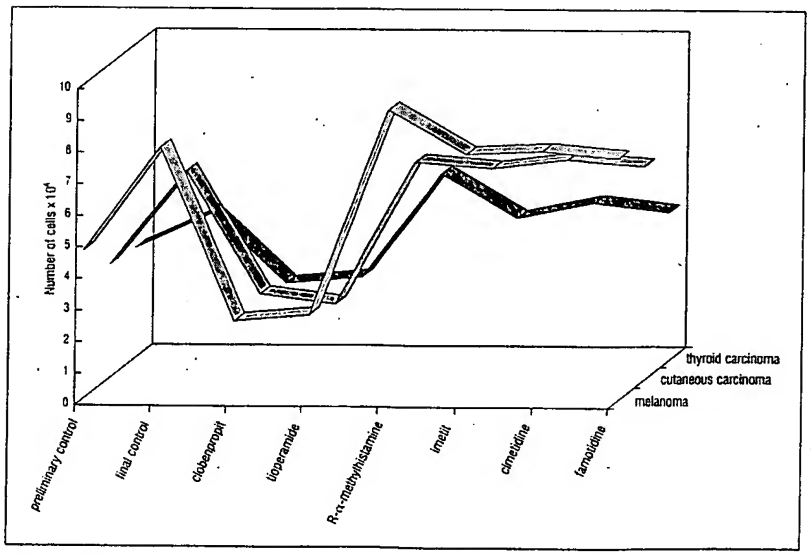

Figure 2. Effect of study modulators of histamine receptors on the number of incubated neoplastic cells (concentration: 200 μg/cm³ each).

Table 3. Effect of study modulators of histamine receptors on the results of proliferative test of human cutaneous carcinoma cells.

Compound	Concentration μg/cm³	WT	IT
		%	
Clobenpropit	200	–30.0	–16.4
	100	6.7	3.6
Tioperamide	200	–40.0	–21.8
	100	–30.0	–16.4
R-α-methylhistamine	200	110.0	60.0
	100	103.3	56.4
Imetit	200	96.7	52.7
	100	103.3	56.4
Cimetidine	200	113.3	61.8
	100	96.7	52.7
Famotidine	200	106.7	58.2
	100	113.3	61.8

Table 4. Effect of study compounds upon proliferative test results of cells of human medullary thyroid carcinoma.

Compound	Concentration μg/cm³	WT	IT
		%	
Clobenpropit	200	–110.0	–83.7
	100	–27.3	–20.8
Tioperamide	200	–81.8	–62.3
	100	–18.2	–13.8
R-α-methylhistamine	200	218.2	166.0
	100	181.8	138.4
Imetit	200	90.9	69.2
	100	127.3	96.8
Cimetidine	200	136.4	103.8
	100	163.4	124.5
Famotidine	200	109.1	83.0
	100	109.1	83.0

Tioperamide — with the strength corresponding to its concentration — demonstrated tendency to inhibit proliferation of the cells of human cutaneous carcinoma. TI values were 21.8 at 200 μg/cm³ and 16.4 at 100 μg/cm³ (table 3).

The remaining study compounds — R-alpha-methylhistamine, imetit, cimetidine and famotidine did not inhibit the proliferation of human cutaneous carcinoma cells, but they even increased this proliferation (figure 2). It was only clobenpropit in the dose of 200 μg/cm³ that slightly inhibited the proliferation of these cells (table 3).

Antagonists of histamine H_3 receptors, clobenpropit and tioperamide inhibited the proliferation of cells of human medullary thyroid carcinoma with the strength adequate to their concentration. Test indices (TI) were –83.7 and –62.3, respectively in the case of 200 μg/cm³, and –20.8 and –13.8 in the case of 100 μg/cm³ (table 4).

R-alpha-methylhistamine and imetit — agonists of histamine H_3 receptors — as well as cimetidine and famotidine — antagonists of histamine H2 receptors increased cell proliferation of human medullary thyroid carcinoma. TI values were 166.0, 69.2, 103.8, 83.0 respectively in the case of 200 μg/cm³ and 138.4, 96.8, 124.5, 83.0 in the case of 100 μg/cm³ of each compound (table 4).

Excluding R-alpha-methylhistamine, no correlation between the activity of compounds and their concentration was observed when cell proliferation of this neoplasm increased (table 4).

An earlier incubation of the cells of the following human neoplasms: melanoma, cutaneous carcinoma and medullary thyroid carcinoma with 200 μg/cm³ tioperamide weakened proliferation-increasing activity of R-alpha-methylhistamine used in the same concentration (figure 3). TI values equal 24.8, 33.6, 38.7 (table 5).

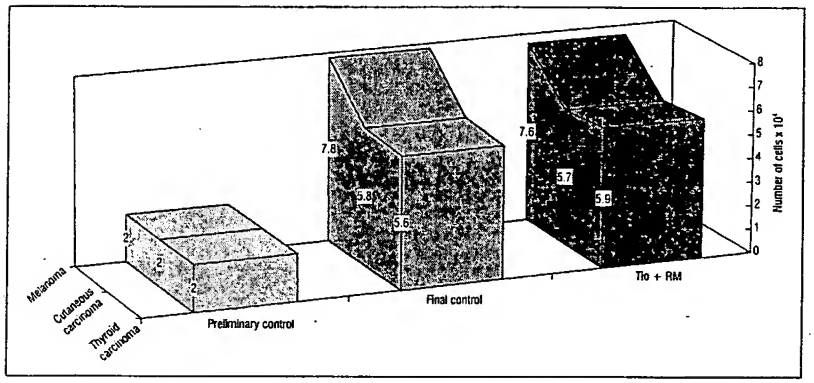

Figure 3. Effect of tioperamide on proliferative action of R-alpha-methylhistamine (RM).

Table 5. Effect of tioperamide (T) on the activity of R-α-methylhistamine (RM) and clobenpropit (C), on the activity of imetit (I), in the concentrations of 200 μg/cm³ each, in proliferative test.

Type of neoplasm	T+RM		C+I	
	WT	IT	WT	IT
	%			
Human melanoma	93.6	24.8	24.1	6.2
Cutaneous carcinoma	97.4	33.6	26.3	9.1
Medullary thyroid carcinoma	108.3	38.7	33.3	11.9

Likewise, an earlier incubation of the cells of human neoplasms listed above with 200 μg/cm³ clobenpropit reduced the proliferation-increasing activity of imetit applied in the same concentration (figure 4). TI values are 6.2, 9.1 and 11.9 (table 5).

DISCUSSION

The antagonists of histamine H_3 receptors tioperamide and clobenpropit have a very clear inhibiting effect upon the proliferation of human neoplastic cells of melanoma, cutaneous carcinoma and, to a slightly lesser extent — thyroid carcinoma. An opposite effect is observed in the case of histamine H_3 receptor agonists — R-alpha-methylhistamine and imetit, which very clearly stimulate the proliferation of these cells.

Interestingly, non-neoplastic cells — of bovine thyroid, are not very sensitive to inhibiting activity of antagonists and proliferation-stimulating activity of agonists of histamine H_3 receptors.

These observations suggest that mediators released after the administration of the antagonists of these receptors, or intracellular changes that take place after their administration inhibit or stimulate the proliferation of neoplastic cells, but not of normal cells.

The fact that cutaneous neoplasms and melanomas are the most sensitive to their effect points to a considerable role of histamine; perhaps the fact that skin contains large amounts of this amine also has some significance.

As mentioned in the introductory section of this work, histamine reveals proliferative properties — it speeds up the process of wound healing as well as the development of certain neoplasms.

The present study confirms the undeniable role of histamine in the proliferation of cells but it also suggests that histamine increases cell proliferation through H_3 receptors being their natural agonist since increased cell proliferation is caused by histamine [18,19], as well as R-α-methylhistamine and imetit — H_3 receptor agonists.

Because H_3 receptor antagonists inhibit the proliferative activity of H_3 receptor agonists, it may be claimed that this effect is associated with H_3 receptors. It also seems true that perhaps not the histamine itself plays a role in cell proliferation but rather intracellular changes that take place after modulation of H_3 receptors. This claim is suggested by the main mechanism of action of study compounds. The antagonists of these receptors (tioperamide and clobenpropit) inhibit cell proliferation, but they enhance the release of histamine, while agonists (R-alpha-methylhistamine and imetit) increase cell proliferation but they inhibit histamine release.

If proliferation depended solely on histamine, the activity of modulators of histamine H_3 receptors should be just the opposite.

Inhibition of cell proliferation in vitro does not have to predetermine in vivo activity. At first, the neoplasm is not vascularised and its alimentation takes place only through diffusion. The development of vascular network takes place later on and

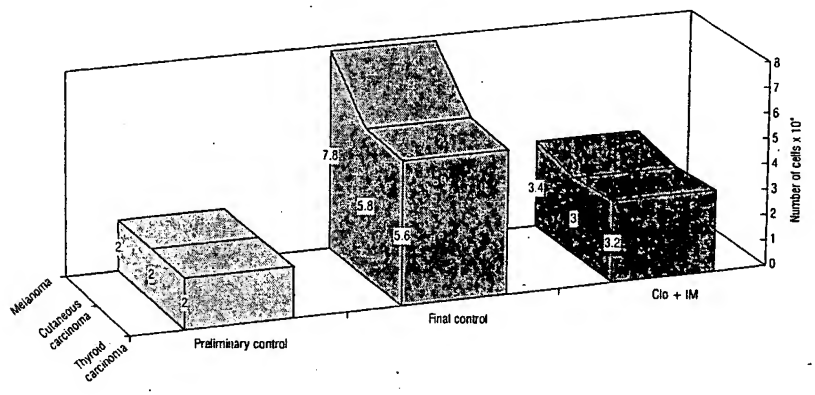

Figure 4. Effect of clobenpropit (Clo) on proliferative action of imetit (IM) — concentration 200 μg/cm³ each.

it is related to the production of tumour angiogesic factor (TAF) — a substance stimulating the proliferation of endothelium, which is probably of no significance in in vivo culture [20,21].

Apart from exogenous factors, the proliferation of neoplastic cells is also determined by endogenous substances such as certain hormones, toxins of intestinal bacteria, nitrocompounds, polyamines, eicosanoids [21]. In the experiment described in the present work — in vitro culture — polyamines and eicosanoids may play an important role, while the significance of other endogenous substances can be excluded.

Prostaglandins as well as other metabolites of arachidonic acid take part in the proliferation of normal and neoplastic cells [18]. Large amounts of eicosanoids were found in human neoplasms (mammary, bronchial, renal and thyroid carcinoma) as well as in experimental neoplasms in animals (mammary carcinoma, sarcoma in mice). Thromboxane B_2 [21] stimulates the growth of melanoma in vitro in mice. Large amounts of prostaglandins produced within neoplasm may inhibit immune reactions of the body and facilitate metastases formation [22]. It was also demonstrated that histamine increases the adhesion of eosinophils to epithelium cells of airways in guinea pig (a rich source of prostaglandins); this effect is inhibited by indomethacin [23].

As far as experimental neoplasms are concerned, e.g. Morris and Walker hepatoma as well as other actively-proliferating neoplastic cells, the level of polyamines is a few times higher than in the cells from which these neoplasms originated. Slowly growing hepatomas contain smaller amounts of polyamines. It was also observed that there is a large amount of polyamines in human neoplastic cells (e.g. sarcomas, hepatomas, lymphatic leukaemias) [21].

Available literature does not abound in data concerning the effect of H_3 receptor modulation upon the synthesis and secretion of polyamines and eicosanoids.

The interrelationships between histamine and polyamines (amine structure, similar synthesising enzymes, similar structure of compounds inhibiting their synthesis) [24] suggest that histamine may play a role in the synthesis of polyamines [25,26] and hence, in the proliferation of cells.

It was found [3] that 1-2 hours after i. p. administration of tioperamide to rats, the production of histamine and the activity of ornithine decarboxylase increases in intestinal mesenteric nodes [3]. It was also proved that increased activity of ornithine decarboxylase is inhibited by H_1 receptor antagonists after its oral administration. The authors of this work suggest that through interaction with H_1 — but not H_2 receptors, histamine increases the activity of ornithine decarboxylase (and consequently, also the synthesis of polyamines which stimulate cell proliferation) [3]. This property of histamine — increasing the activity of ornithine decarboxylase — is beneficial in the case of regeneration of damaged cells, as histamine speeds up reconstruction processes in tissues, but it is also undesirable in the case of neoplastic cells as histamine increases their proliferation and speeds up the development of neoplasms.

The recent in vitro studies on cell cultures of gastric carcinoma Kato — III and AGS revealed that histamine increases the proliferation of these cells (in maximum concentration 10^{-5} M) with coexisting increased activity of ornithine decarboxylase. This effect of histamine was reversed by 10^{-5} M cimetidine, while the activity of ranitidine and famotidine was doubtful [19]. The authors of these studies suggest that cimetidine has an antiproliferative effect on the development of examined cells of gastric carcinoma. It seems however, that increased polyamine formation observed by these authors (increased activity of ornithine decarboxylase) indicates that their inhibition does not play an important role in this mechanism [19] as higher level of polyamines is responsible for increased proliferation and not for its inhibition.

In my study, there was no evidence for an antiproliferative activity of cimetidine or famotidine upon cell cultures of human medullary thyroid carcinoma, melanoma and cutaneous carcinoma.

In the absence of antiproliferative effect of ranitidine and famotidine, it seems that the blockade of H_2 receptors does not play any role in the mechanisms of antiproliferative activity of cimetidine suggested by others.

Cimetidine is also an agonist of imidazoline receptors (I_2), but there are no data concerning interrelationships between this receptor and proliferation of cells. It is a fact that cimetidine is a known immunostimulator [27].

Similar imidazole chemical structure of histamine, tioperamide and clobenpropit may suggest their possible action through imidazole receptors, but to-date there are no data concerning this issue.

Final remarks

Antagonists of histamine H_3 receptors (tioperamide and clobenpropit) inhibit the proliferation of examined neoplastic cells, while agonists of these receptors (R-alpha-methylhistamine and imetit) have a histamine-like effect, i.e. they increase the proliferation of neoplastic cells.

As H_3 receptor antagonists inhibit proliferative action of H_3 receptor agonists, it may be presumed that effect on proliferation depends upon the effect on these receptors.

The results of these studies also suggest that proliferative action of histamine, which was proved by other authors, and its so-far unknown mechanism, may be related to the stimulation of H_3 receptors by histamine, as the latter is a natural agonist of these receptors and it has an identical effect upon proliferation as observed in the case of selective agonists — R-alpha-methylhistamine and imetit.

Neither cimetidine, nor famotidine have an antiproliferative activity on cell cultures of human neoplasms: medullary thyroid carcinoma, melanoma and cutaneous carcinoma.

The findings reported in the present work suggest that antagonists of histamine H_3 receptors might play some role inhibiting the growth of certain neoplasms, and agonists of these receptors might be used — just as histamine — in order to increase proliferation of cells, e.g. of skin (in vitro cultures for transplantation); however, it would be premature to speculate about practical therapeutic implications.

CONCLUSIONS

1. Antagonists of histamine H_3 receptors (tioperamide and clobenpropit) inhibit the proliferation of human neoplastic cells — melanoma, cutaneous carcinoma and medullary thyroid carcinoma.
2. Agonists of histamine H_3 receptors increase cell proliferation of examined human neoplasms.
3. The cells of normal bovine thyroid are slightly sensitive to proliferation-inhibiting activity of antagonists and proliferation-stimulating activity of agonists of H_3 receptors.
4. Antagonists of H_3 receptors inhibit the proliferative activity of H_3 receptor agonists, therefore, it may be claimed that this action is related to H_3 receptors.

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Histamine H_3 Antagonists

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Summary

Treatment of allergic rhinitis often involves the use of H_1 antihistamines which block histamine, a primary mediator in allergic responses. Antihistamines alone do not provide significant benefit against the congestion associated with allergic rhinitis and are commonly given in combination with α-adrenergic decongestants. Whereas these decongestants are effective in reducing the congestion associated with allergic nasal disease, they may produce undesirable side effects, such as hypertension, agitation and insomnia. Presently, we discuss preclinical findings showing that combination histamine H_1 and H_3 receptor blockade produces decongestant activity without the hypertensive liability characteristic of α-adrenoceptor agonists.

Allergic rhinitis is an inflammation of the nasal mucosa characterized by nasal congestion and rhinorrhea. This allergic nasal disease has been estimated to occur in up to 22% of the US population [1]. Untreated rhinitis often leads to sinusitis, otitis media, nasal polyps and aggravation of bronchial asthma [2]. In the nose, mast cell histamine mediates the immediate hypersensitivity reactions that occur secondary to antigen exposure in sensitized individuals [3]. Histamine is a primary mediator responsible for the increases in nasal mucus secretion, rhinorrhea, sneezing and pruritis found in allergic rhinitis [4]. Although antihistamines are the primary treatment for allergic rhinitis, present findings indicate that the antiallergy efficacy of antihistamines against mast-cell-mediated nasal congestion may be expanded by cotreatment with a histamine H_3 receptor antagonist [5].

Antihistamines and α-Adrenergic Nasal Decongestants

Many of the allergic effects of histamine are attenuated by first- and second-generation H_1 blockers [6]. However, antihistamines are often used in combination with α-adrenergic agonist decongestants because the congestion associated with allergic rhinitis is refractory to H_1 antihistamines [7]. The use of α-agonists is limited, however, due to their potential to produce hypertensive and CNS-stimulant effects.

Modulation of Autonomic Responses by Histamine H_3 Receptors

The discovery by Arrang et al. [8] of a unique histamine H_3 receptor has rekindled interest in exploring the role of histamine and the potential for a new class of therapeutic agents that act at this receptor. Histamine H_3 receptors are widely distributed on peripheral autonomic nerves, and are found presynaptically on postganglionic nerve terminals. Activation of these receptors inhibits neurally induced cholinergic contraction in guinea pig trachea [9], human bronchus [10] and guinea pig ileum [11, 12]. They are also found presynaptically on postganglionic sympathetic nerves, and activation attenuates a variety of effector responses [13, 14]. Specifically, H_3 receptors modulate vascular responses by inhibition of norepinephrine release from sympathetic nerve terminals [15–17].

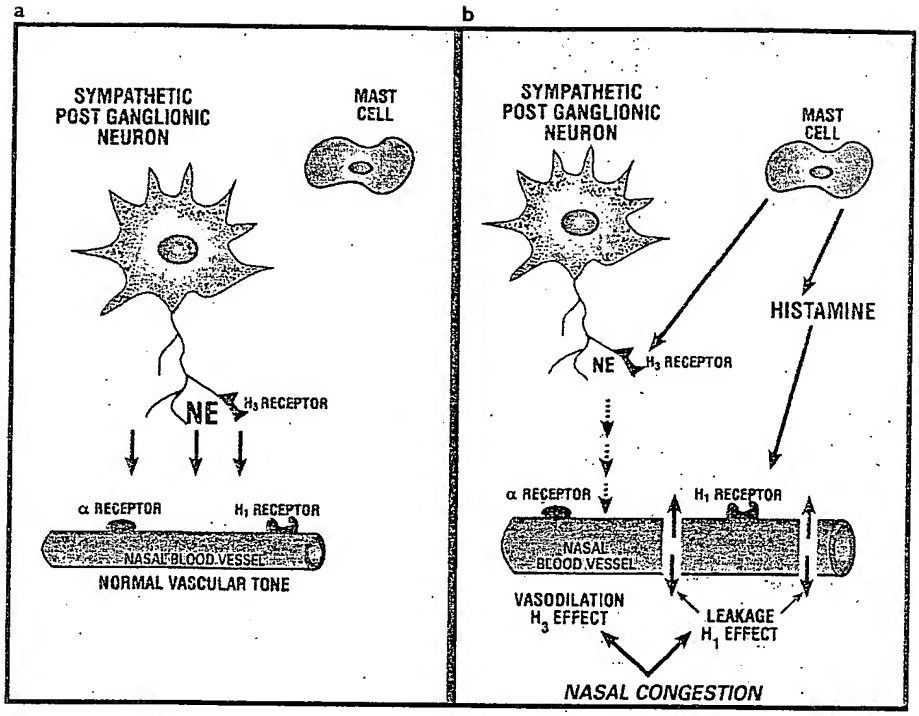


Fig. 1. Proposed mechanism for the nasal decongestant effect of combined histamine receptor H_1/H_3 blockade. **a** Nasal vascular tone is under autonomic sympathetic control mediated by tonic release of norepinephrine (NE) from postganglionic sympathetic neurons. **b** During an allergic reaction, combined H_1/H_3 treatment blocks both the direct vascular effects (plasma extravasation) of mast cell histamine and the vasodilation that occurs secondary to inhibition of sympathetic tone mediated by histamine-induced activation of presynaptic H_3 receptors.

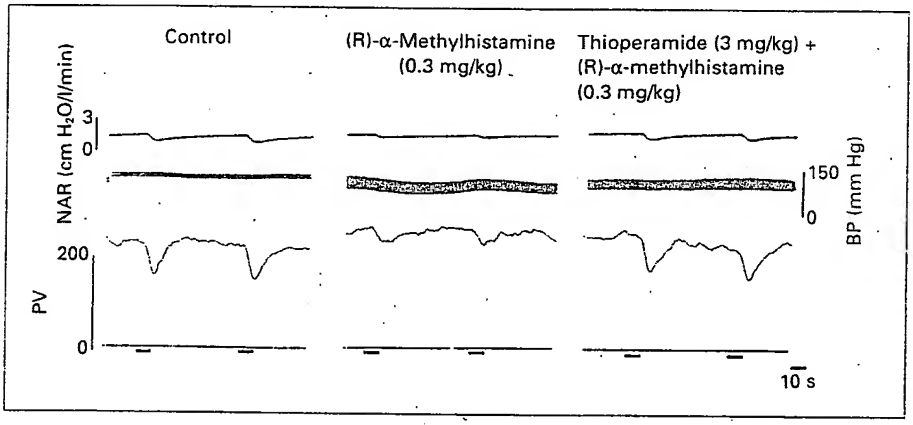


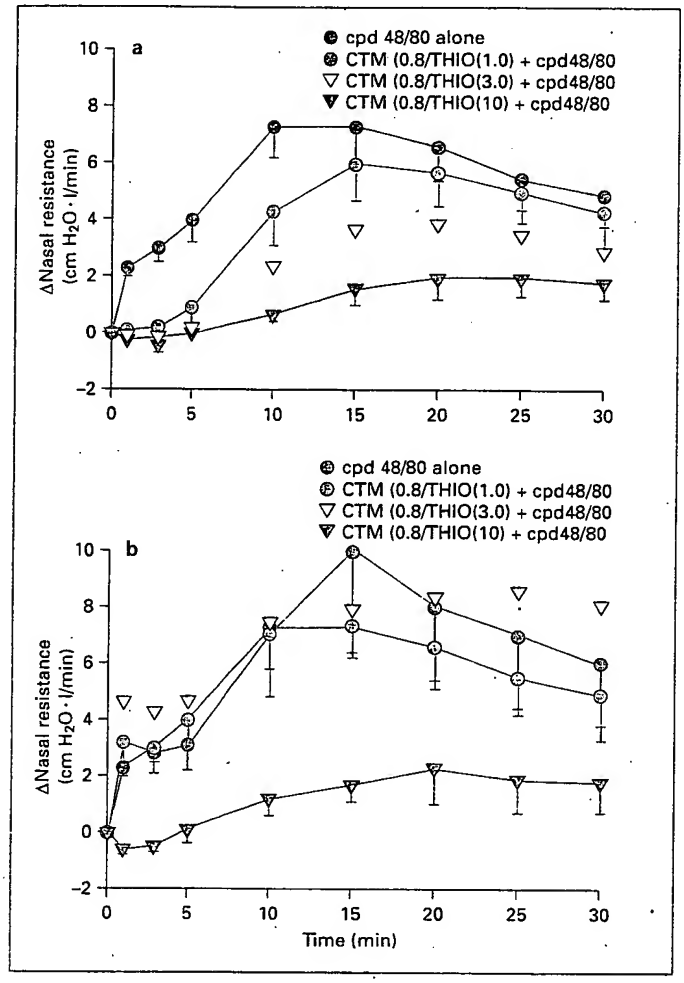
Fig. 2. Effect of histamine H_3 receptor activation on sympathetic modulation of nasal blood flow (PV = perfusion value, an index of blood flow measured by laser Doppler) and nasal resistance (NAR). Shown on the chart recordings are the effects of (*R*)-α-methylhistamine on sympathetic-mediated decreases in nasal resistance and nasal blood flow. BP = Blood pressure.

Rationale for Combined Histamine H_1/H_3 Treatment for Allergic Rhinitis

In the nose, sympathetic modulation of nasal blood flow is a regulator of nasal patency. Because antihistamines do not block all the nasal effects of histamine, we studied the nasal effects of combined histamine H_1/H_3 blockade in an experimental model of nasal congestion. Under quiescent conditions, nasal vascular tone is maintained by continuous sympathetic regulation of nasal blood vessel patency (fig. 1a). After mast cell degranulation produced by an allergic reaction or by compound 48/80 as in our studies, released histamine acts on postsynaptic H_1 receptors to cause H_1-mediated plasma extravasation, vasodilation and mucus secretion. In addition, histamine produces vasodilation by activating presynaptic H_3 receptors located on postganglionic sympathetic neurons producing a decrease in norepinephrine release (fig. 1b). Combined dual H_1/H_3 treatment blocks both H_1- and H_3-mediated consequences of mast cell histamine liberation.

Histamine H_3 Modulation of Nasal Blood Flow and Nasal Resistance

Recent studies have shown a physiological role for peripheral sympathetic presynaptic H_3 receptors on regulation of nasal vascular tone. Bolser et al. [18] found that activation of histamine H_3 receptors inhibits the decrease in nasal blood flow and nasal resistance elicited by electrical stimulation of the cervical sympathetic trunk in cats. Figure 2 displays a typical effect of (*R*)-α-methylhis-



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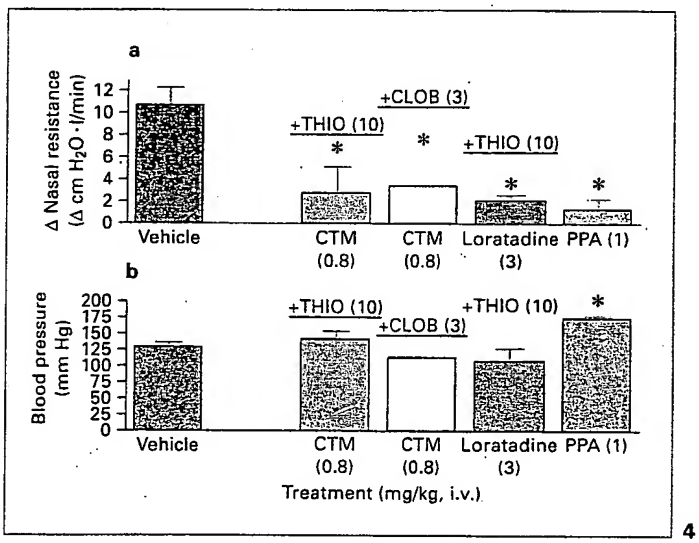

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Fig. 3. Effect of combined histamine H_1/H_3 treatment with chlorpheniramine (CTM, 0.8 mg/kg, i.v.) and thioperamide (THIO 1.0–10 mg/kg, i.v.) on aerosolized compound 48/80 (1%)-induced increase in nasal resistance. Each point represents the mean ± SEM of 4–7 animals (* $p < 0.05$ compared to compound 48/80 alone).

Fig. 4. Effect of combined histamine H_1/H_3 treatment on nasal resistance and blood pressure. **a** Effect of chlorpheniramine (CTM; 0.8 mg/kg, i.v.) plus thioperamide (THIO; 10 mg/kg, i.v.); CTM (0.8 mg/kg, i.v.) plus clobenpropit (CLOB; 3 mg/kg, i.v.); loratadine (3 mg/kg, i.v.) plus THIO (10 mg/kg, i.v.) and phenylpropanolamine (PPA; 1 mg/kg, i.v.) on the increase in nasal resistance produced by aerosolized compound 48/80 (1%). **b** Effect of various H_1/H_3 combinations on blood pressure. Each point represents the mean ± SEM of 4–7 animals (* $p < 0.05$ compared to vehicle).

tamine (0.3 mg/kg, i.v.), an H_3 agonist, on nasal blood flow and resistance. In these studies, (*R*)-α-methylhistamine inhibited the sympathetic-mediated decrease in blood flow and nasal resistance by 49 ± 11 and 34 ± 11%, respectively. The activity of (*R*)-α-methylhistamine was blocked by the H_3 antagonist thioperamide (3.0 mg/kg, i.v.).

Evaluation of the Nasal Decongestant Activity of Combined H_1/H_3 Blockade

We evaluated the effect of combined H_1 and H_3 receptor blockade on the increases in nasal resistance produced by nasal provocation with aerosolized compound 48/80 in the cat [5, 19]. Figure 3 illustrates the decongestant effect of dual histamine H_1/H_3 receptor blockade on compound 48/80 (1%)-induced nasal responses. Chlorpheniramine (0.8 mg/kg, i.v.) plus thioperamide (0.1–10 mg/kg, i.v.) given before nasal exposure to compound 48/80 produced a dose-dependent inhibition of nasal resistance. Similar results were obtained with clobenpropit, an H_3 antagonist that is structurally different from thioperamide. The combination of clobenpropit (3.0 mg/kg, i.v.) and chlorpheniramine (0.8 mg/kg, i.v.) also produced nasal decongestion (fig. 4). Moreover, the nonsedating antihistamine, loratadine (3.0 mg/kg, i.v.) plus thioperamide (10 mg/kg, i.v.) inhibited the nasal congestion due to compound 48/80 (fig. 4). When evaluated separately, the H_3 antagonists thioperamide (10 mg/kg, i.v.) and clobenpropit (3.0) or the H_1 antagonists chlorpheniramine (3.0 mg/kg, i.v.) or loratadine (3.0 mg/kg, i.v.) did not produce decongestion (fig. 3b; clobenpropit and loratadine data not shown). Run as a positive standard, the α-agonist phenylpropanolamine (1.0 mg/kg, i.v.) also blocked the congestive actions of compound 48/80 while significantly increasing blood pressure (fig. 4). In contrast to the hypertension produced by phenylpropanolamine, dual histamine H_1/

Table 1. Effect of oral histamine H_1/H_3 blockade on nasal cavity geometry and blood pressure

Treatment, mg/kg, p.o.	n	Decrase in nasal cavity volume, %	Systolic BP mm Hg
Compound 48/80 alone	8	68 ± 8	89 ± 8
CTM (10)/THIO (30) + cpd 48/80	6	19 ± 12*	81 ± 8
PPA (10) + cpd 48/80	6	18 ± 9*	138 ± 8*
CTM (10) + cpd 48/80	7	64 ± 14	81 ± 6
THIO (30) + cpd 48/80	5	40 ± 10	89 ± 7

* $p < 0.05$, statistically different from control animals given compound 48/80 (cpd 48/80) alone.

H_3 blockade with chlorpheniramine plus thioperamide, chlorpheniramine plus clobenpropit, or loratadine plus thioperamide did not affect blood pressure (fig. 4).

Combined oral H_1/H_3 treatment was also found to attenuate the decrease in nasal cavity volume produced by topical exposure to compound 48/80 (1%, 50 µl). Using acoustic rhinometry, we evaluated the nasal decongestant activity of chlorpheniramine (10 mg/kg, p.o.) administered in combination with thioperamide (30 mg/kg, p.o.) on nasal cavity geometry (table 1). The nasal volume data are expressed as the percent decrease in nasal cavity volume from a naive group of animals (no compound 48/80 exposure) 3 h after oral treatments. Phenylpropanolamine (10 mg/kg, p.o.) also produced nasal decongestion. Treatment with chlorpheniramine (10 mg/kg, p.o.) or thioperamide (30 mg/kg, p.o.) alone or together had no effects on systolic blood pressure, while phenylpropanolamine (10 mg/kg, p.o.) produced an increase in blood pressure.

Conclusions

Our results suggest that combined H_1/H_3 blockade may provide a novel approach for the treatment of allergic nasal congestion without the hypertensive liability of current α-adrenergic agonist decongestant therapies. In allergic nasal disease, mast-cell-derived histamine in addition to producing H_1-receptor-mediated effects, such as plasma extravasation, sneezing and mucus secretion, may also inhibit sympathetic outflow producing nasal vasodilation by a prejunctional H_3 receptor mechanism. In conclusion, the addition of an H_3 antagonist to an H_1 antagonist confers decongestant activity upon the H_1 blocker by attenuating autonomic effects of histamine not blocked by antihistamines.

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Combined Histamine H_1 and H_3 Receptor Blockade Produces Nasal Decongestion in an Experimental Model of Nasal Congestion

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ABSTRACT

We studied the pharmacological actions of combined histamine H_1/H_3 receptor blockade on the increase in nasal airway resistance (NAR) and decrease in nasal cavity volume produced by nasal exposure to compound 48/80, a mast cell degranulator. In the anesthetized cat compound 48/80 (1%) produced a maximum increase in NAR of 9.1 ± 0.7 cmH_2O·L/minute. The increase in NAR in animals pretreated with a combination of the H_1 antagonist, chlorpheniramine (CTM; 0.8 mg/kg i.v.) and increasing doses of the H_3 antagonist, thioperamide (THIO; 1.0, 3.0, and 10.0 mg/kg i.v.) were 6.1 ± 2.1, 4.2 ± 1.0 and 2.2 ± 0.7 cmH_2O·L/minute, respectively. A second H_3 antagonist, clobenpropit (CLOB; 0.03, 0.3, and 1.0 mg/kg i.v.) combined with CTM (0.8 mg/kg i.v.) also inhibited the nasal effects of compound 48/80. When the nonsedating H_1 antihistamine, loratadine (3.0 mg/kg i.v.), was substituted for CTM, it also reduced nasal congestion when given in combination with THIO (10 mg/kg i.v.). In contrast, treatment with CTM (1.0 mg/kg i.v.) and the H_2 antagonist, ranitidine (RAN; 1.0 mg/kg i.v.) were without activity. Loratadine, CTM, CLOB, RAN, or THIO administered alone were inactive. The α-adrenergic agonist, phenylpropanolamine (PPA; 1.0 mg/kg i.v.) demonstrated decongestant effects, but in contrast to H_1/H_3 blockade, PPA produced a significant hypertensive effect. Using acoustic rhinometry (AcR) we found that combined i.v. CTM (1.0 mg/kg) and THIO (10 mg/kg) and combined oral CTM (10 mg/kg) and THIO (30 mg/kg) blocked the decrease in nasal cavity volume produced by intranasal compound 48/80 (1%, 50 μL). We conclude that combined H_1/H_3 histamine receptor blockade enhances the efficacy of an H_1 antagonist by conferring decongestant activity to the H_1 antihistamine. We propose that the decongestant activity of combined H_1/H_3 blockade may provide a novel approach for the treatment of allergic nasal congestion without the hypertensive liability of current therapies. (American Journal of Rhinology 13, 391–399, 1999)

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Investigations into the pathophysiologic actions of histamine identify this autacoid as a potent mediator of allergic and inflammatory reactions. Histamine is synthesized and stored in secretory granules of mast cells in a variety of tissues throughout the body such as skin, lung, gut, and the lining of the blood vessels.[1] Mast cell histamine is a mediator in immediate hypersensitivity reactions secondary to antigen exposure in sensitized tissues.

After its release, histamine, as well as other mediators, can exert numerous local and systemic effects that contribute to the pathophysiology of allergic rhinitis, urticaria, allergic conjunctivitis, asthma, and anaphylactic shock.[2] Specifically, histamine contributes largely to early phase bronchospasms, evokes mucus secretion, increases vascular permeability, and induces pruritis. Historically, the actions of histamine has been ascribed to activation of histamine H_1 and H_2 receptors with H_1 effects predominating, because many of these actions can be blocked by classic sedating and second generation (nonsedating) antihistamines.[3,4] Antihistamines are indicated and widely used for the management of allergic diseases.[5–10]

Although H_1 antihistamines are the mainstay treatment in allergic diseases, there are significant limitations to their use. First generation histamine H_1 antagonists, such as diphenhydramine and promethazine, have significant sedative and anticholinergic activity in man that limits their clinical usefulness.[4] Newer second generation H_1 antihistamines, such as loratadine and terfenadine, are nonsedating and completely devoid of CNS effects. Although the newer nonsedating antihistamines like loratadine have become the preferred first line treatment of allergic rhinitis, they are often used in combination with α-adrenergic agonist decongestants because the antihistamines alone do not block the nasal congestion that accompanies allergic rhinitis. Also, the use of α-adrenoreceptor agonists may be limited by their potential systemic cardiovascular and CNS stimulant effects.

Histamine H_3 receptors are found on sympathetic nerves, where they modulate sympathetic neurotransmission[11,12] and attenuate a variety of effector organ responses under control of the sympathetic nervous system.[13–15] Specifically, histamine H_3 receptor activation modulates outflow to resistance and capacitance vessels causing vasodilatation.[16–18] Recently Hey et al. (1998) have shown that stimulation of H_3 receptors modulate sympathetic regulation of nasal resistance. Therefore, release of mast cell-derived histamine during an allergic reaction may contribute to nasal congestion through activation of prejunctional H_3 receptors, which would result in nasal vascular engorgement (Fig. 1).

Because not all the actions of histamine are blocked by H_1 antihistamines, the focus of the current studies was to determine whether an H_3 antagonist, when given in combination with an H_1 antagonist, confers decongestant efficacy against nasal congestion produced by histamine released from mast cells by compound 48/80. The present studies show using two different methods, namely, pressure rhinometry and acoustic rhinometry, that combined H_1/H_3 receptor blockade produces nasal decongestion without effects on systemic blood pressure.

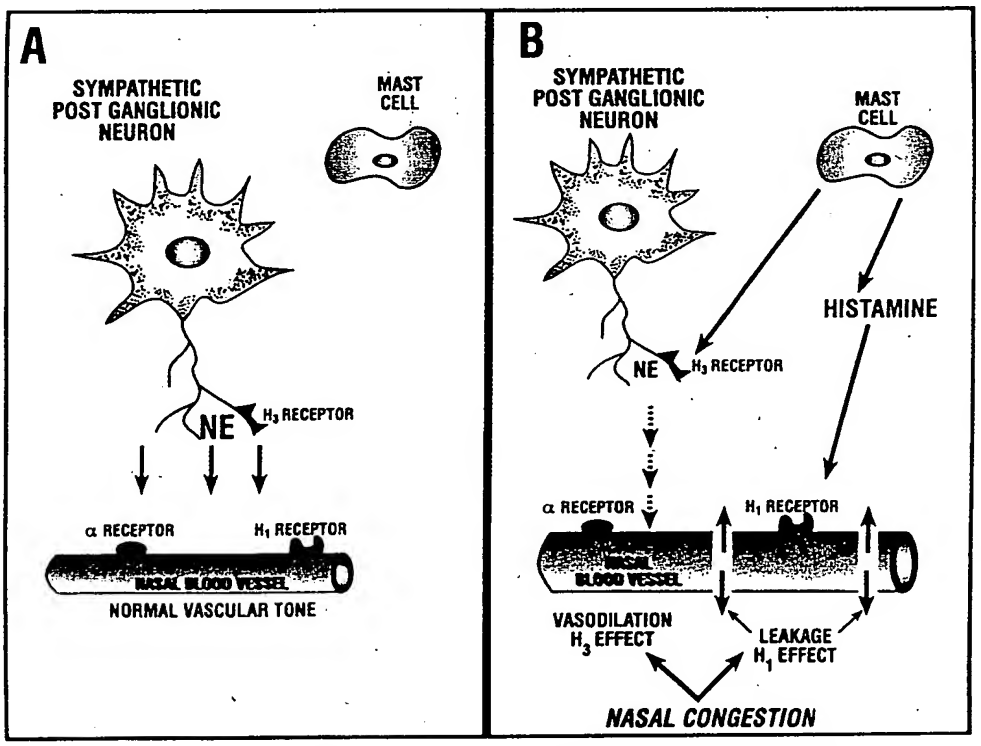

Figure 1. Proposed mechanism of nasal decongestant action of combined H_1/H_3 receptor blockade. (A) Under quiescent conditions, nasal vascular tone is maintained in part by continuous adrenergic sympathetic regulation of nasal blood vessels patency. After mast cell degranulation produced by allergic reaction, or by compound 48/80 as in the present study, released histamine acts at postsynaptic H_1 receptors to cause H_1 mediated plasma extravasation (vasodilation and mucus secretion not shown in diagram). (B) In addition, histamine produces vasodilation by activating presynaptic H_3 receptors located on postsympathetic ganglionic neurons and decreasing norepinephrine release. Combined dual H_1/H_3 treatment blocks both H_1 and H_3 mediated consequences of mast cell histamine liberation.

MATERIALS AND METHODS

General

In all experiments, male cats (1.5–3.0 kg, Harlen Sprague–Dawley, Madison, WI) were anesthetized with pentobarbital (35 mg/kg i.p.). For nasal airway resistance (NAR) studies, the right femoral artery and vein were cannulated for measurement of blood pressure and administration of drugs, respectively.

Measurement of Nasal Airway Resistance in the Cat

The method for evaluating decongestant activity in anesthetized cat has been previously described.[19] Briefly, anesthetized cats were paralyzed with pancuronium bromide (5 mg/kg i.v.) and mechanically ventilated (volume = 25 mL, rate = 20 breaths/minute) with room air. A cuffed endotracheal tube was advanced retrograde from the upper esophagus to the nasopharynx. The tube was inflated to form an airtight fit. The left nostril was sealed with reprosil (Dentsply International Inc., Milford, DE). Air was passed through the right nasal airway via the endotracheal tube at a constant rate of 1.7 L/minute. Pressure across the nasal cavity, blood pressure, and pulmonary insufflation pressure were derived using physiologic pressure transducers (P23XL, Viggo-Spectramed, Oxnard, CA) and were recorded continuously on a Grass chart recorder.

Measurement of Nasal Cavity Volume Using Acoustic Rhinometry

Measurements of nasal minimum cross-sectional areas (A_{min}) and estimates of nasal volumes were determined in the anesthetized cat according to the methods of McLeod et al.[19] AcR equipment was purchased from NADAR (Aarhus, Denmark). Cats were placed in a supine position. A spark generated sound wave was dispersed down a rigid wave tube and entered the nasal cavity through a plastic nose piece. The reflected sound waves from the nose were amplified and recorded. The sampling frequency was 100 KHz. The data obtained were converted to area-distance function curves and were used to provide estimates of cross-sectional areas and nasal volumes. The length of the nose piece was 3.2 cm. The distance measured from the nostril opening into the nasal cavity was 3.0 cm and was based on measurements of cast impressions made of the cat nasal passageways.

Evaluation of Combined Histamine H_1/H_3 Blockade on Nasal Airway Resistance

The nasal decongestant effect of combined histamine H_1/H_3 receptor blockade on NAR responses to aerosolized compound 48/80 (1%, 45 sec), a mast cell mediator releaser[20] were studied. The action of the H_1 antagonist, chlorpheniramine (CTM: 0.8 and 3.0 mg/kg i.v.) and the H_3 antagonist, thioperamide (THIO; 10 mg/kg i.v.), administered alone and a combination administration of CTM (0.8 mg/kg i.v.) with graded doses of THIO (1.0–10 mg/kg i.v.) was evaluated. The dose of CTM (0.8 mg/kg i.v.) used in the current studies was chosen because it inhibited the increase in NAR produced by aerosolized histamine by 50% in this preparation (data not shown). We also studied the pharmacology of dual histamine H_1/H_3 receptor blockade with a second histamine H_3 antagonist, clobenpropit (CLOB), which is structurally different from THIO. In these studies, the effects of combined treatment with CLOB (0.3–3.0 mg/kg i.v.) and CTM (0.8 mg/kg i.v.) were evaluated. A second H_1 antagonist, the nonsedating antihistamine, loratadine (3.0 and 10 mg/kg i.v.) was also evaluated in combination with THIO (10 mg/kg i.v.). In separate studies, the effect of the α-agonist phenylpropanolamine (PPA; 1.0 mg/kg i.v.) on the increase in NAR produced by compound 48/80 was also studied. Finally, the decongestant effect of combined H_1 and H_2 receptor blockade was evaluated using CTM (1.0 mg/kg, i.v.) and the H_2 antagonist, ranitidine (RAN; 1.0 mg/kg i.v.). Drugs were given 10 minutes before compound 48/80 was administered, and the pharmacological effects were observed for 40 minutes. Blood pressure was measured continuously throughout the experiment via direct cannulation of the femoral artery.

Evaluation of Combined Intravenous and Oral Histamine H_1/H_3 Treatment on Nasal Cavity Volume and Minimum Cross-Sectional Area

The nasal decongestant effect of combined histamine H_1/H_3 treatment with CTM and THIO were studied using AcR. The effect of CTM (1.0 mg/kg i.v.) given in combination with THIO (10 mg/kg i.v.) was evaluated on the decreases in nasal cavity volume and A_{min} produced by topical exposure of the nares to compound 48/80 (1%, 50 μL). For the i.v. studies, drugs were given 10 minutes before compound 48/80 exposure. We also studied the oral decongestant activity of CTM (10 mg/kg) and THIO (30 mg/kg) given in combination and of PPA (10 mg/kg) on the nasal congestant effect of compound 48/80. Cats were hand restrained and drugs were orally administered 2 hours before compound 48/80 was given, and the pharmacological effects were observed at 60 minutes after nasal exposure to compound 48/80. Systolic blood pressure was measured 60 minutes after compound 48/80 was given.

Drugs

The compound 48/80 and phenylpropanolamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Thioperamide maleate, clobenpropit dihydrobromide, and ranitidine hydrochloride were purchased from Research Biochemicals International (Natick, MA). Chlorpheniramine maleate and loratadine were synthesized by Schering Plough Research Institute. Drugs were dissolved in physiologic saline (0.9%), and drug doses refer to their respective free bases. Loratadine was dissolved in a vehicle containing 40% ethanol, 30% dimethylsulfoxide (DMSO), and 30% physiologic saline. Control animals were given appropriate vehicle controls.

Statistics

Nasal airway resistance (NAR) was calculated from the equation, NAR = pressure/flow (cmH_2O·L/minute). For acoustic rhinometry studies, nasal volumes were derived by integrating generated area-distance curves a length between 0 and 3 cm into the nasal cavity. A mean from 5–7 curves was used for each integration. The data from the i.v. AcR studies were expressed as the percent decrease from baseline nasal volumes. For the oral AcR studies, the volumes were expressed as the ratio of the volumes of treated nares versus the untreated nares, and only cats whose baseline ratios were between 0.5 and 1.5 were used in the study. Mean blood pressure was mathematically derived from an arterial pulse pressure using the relationship pulse pressure divided by three plus diastolic blood pressure. Statistical significance was evaluated by a one-way ANOVA in conjunction with a Dunnett's two tailed t test. Statistical significance was set at $p < 0.05$.

Animal Care and Use

These studies were performed in accordance to the NIH Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act in an AAALAC-accredited program.

RESULTS

Effect of Histamine H_1/H_3 Receptor Blockade on Nasal Airway Resistance

Mean baseline NAR and blood pressure values were 2.8 ± 0.2 cmH_2O·L/minute and 136 ± 4 mmHg (n = 33), respectively, and were not different between treatment groups. Aerosolized compound 48/80 (1%) produced a maximum increase of 9.1 ± 0.8 cmH_2O·L/minute in NAR (Fig. 2). The effects of dual histamine H_1/H_3 receptor blockade on the increase in NAR produced by compound 48/80 are shown in Figs 2–4. Treatment with CTM (0.8 mg/kg i.v.) and increasing doses of THIO (0.1–10 mg/kg i.v.) produced a dose dependent inhibition of NAR (Fig. 2). When evaluated separately, THIO (10 mg/kg i.v.) and CTM (0.8 and 3.0 mg/kg i.v.) had no effect on the increase in NAR produced by compound 48/80. Combined histamine H_1/H_3 receptor blockade with CTM (0.8 and CLOB (0.03–1.0 mg/kg i.v.) also produced decongestant effects similar to THIO and CTM (Fig. 3). CLOB (1.0 mg/kg i.v.) alone was inactive. Loratadine (3 and 10 mg/kg i.v.) given alone did not inhibit compound 48/80-induced nasal congestion (Fig. 4). However, when combined with THIO (10 mg/kg i.v.) the H_1/H_3 blockade attenuated the congestive effect of compound 48/80. In contrast with the results on H_1/H_3 blockade, combined H_1/H_2 blockade with CTM (1.0 mg/kg i.v.) and RAN (1.0 mg/kg i.v.) had no decongestant activity (Fig. 5).

The dose dependent decongestant effect of PPA in this model has been previously demonstrated.[19] In the present study, PPA (1.0 mg/kg i.v.) attenuated the congestive actions of aerosolized compound 48/80 (Table I). Associated with the decongestant activity of PPA was a significant increase in blood pressure. In contrast to the hypertension produced by PPA, dual histamine H_1/H_3 blockade with CTM plus THIO, CTM plus CLOB, or loratadine plus THIO did not produce significant changes in blood pressure (Table I).

Effect of Intravenous Histamine H_1/H_3 Receptor Treatment on Nasal Cavity Volume

The mean baseline nasal volume and A_{min} for all treatment groups was 0.641 ± 0.046 mL and 0.030 ± 0.002 cm^2 (n = 20), and there was no difference

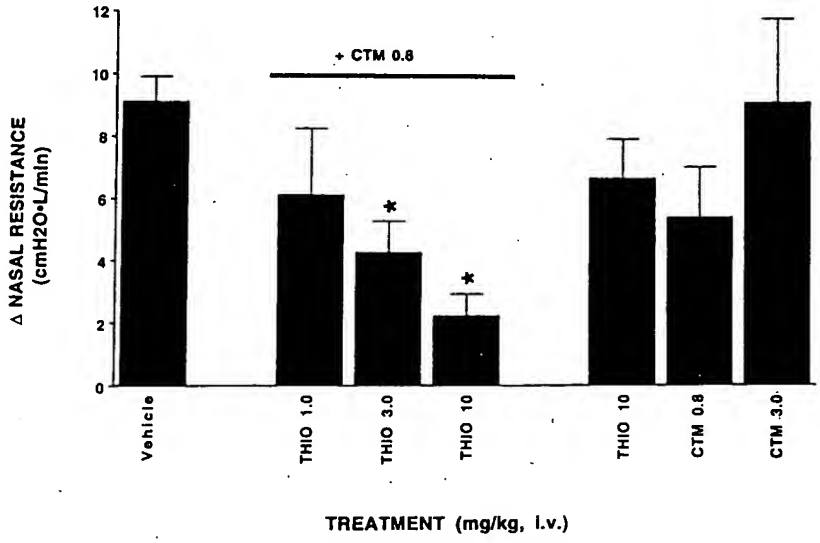

*Figure 2. Effect of chlorpheniramine (CTM) and thioperamide (THIO) treatment on compound 48/80-induced increase in nasal airway resistance (NAR). Shown are the actions of CTM (0.8 mg/kg) combined with graded does of THIO (1.0–10 mg/kg), and CTM (0.8 and 3.0 mg/kg) and THIO (10 mg/kg) given alone compared to vehicle control animals on the increase in NAR due to exposure to aerosolized compound 48/80 (1%). Each bar represents the Mean ± SEM of 4–7 animals (*p < 0.05 compared to vehicle).*

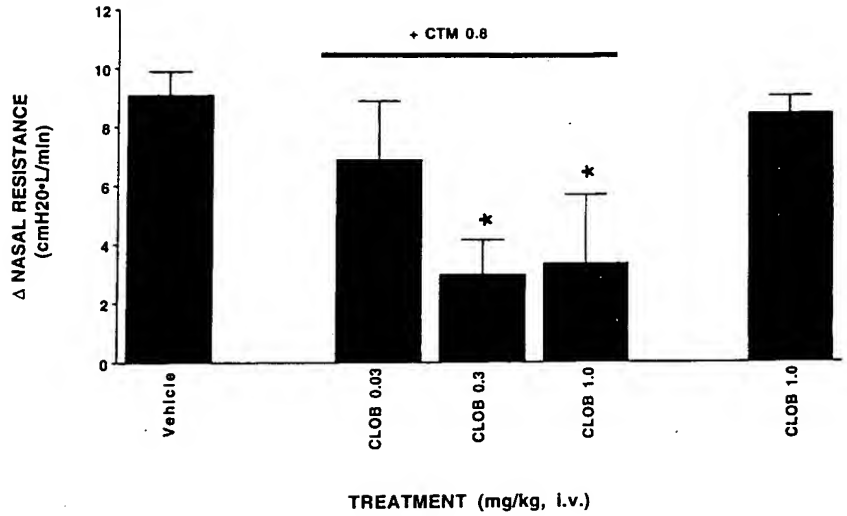

*Figure 3. Effect of chlorpheniramine (CTM) and clobenpropit (CLOB) treatment on compound 48/80-induced increase in nasal airway resistance (NAR). Shown are the actions of CTM (0.8 mg/kg) combined with graded does of CLOB (0.03–1.0 mg/kg), and CLOB (1 mg/kg) given alone compared to vehicle control animals on the increase in NAR due to exposure to aerosolized compound 48/80 (1%). Each bar represents the Mean ± SEM of 5–9 animals (*p < 0.05 compared to vehicle).*

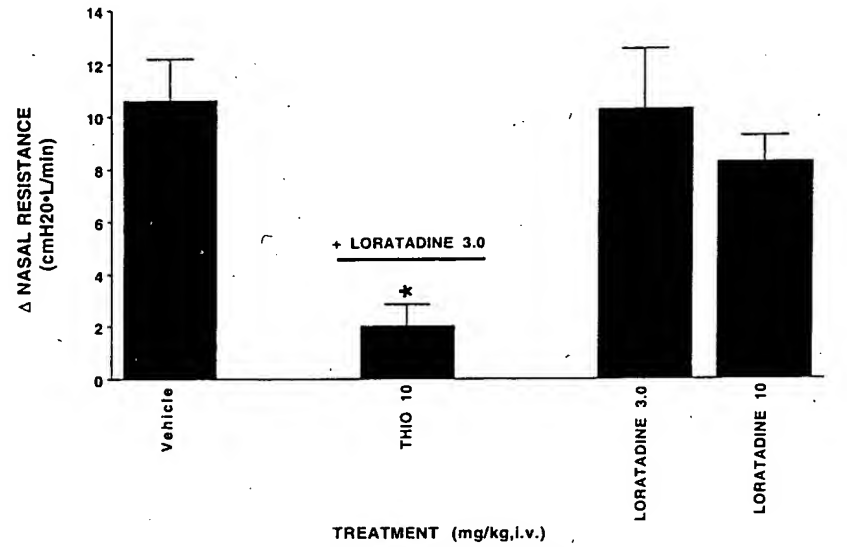

*Figure 4. Effect of loratadine and thioperamide (THIO) treatment on compound 48/80-induced increase in nasal airway resistance (NAR). Shown are the actions of loratadine (3.0 mg/kg) combined with THIO (10 mg/kg), and loratadine (3 and 10 mg/kg) given alone compared to vehicle control animals on the increase in NAR due to exposure to aerosolized compound 48/80 (1%). Each bar represents the Mean ± SEM of 6–10 animals (*p < 0.05 compared to vehicle).*

among the groups. Compound 48/80 (1%) given into the left nasal cavity decreased nasal cavity volume from baseline values by 59 ± 10% (Fig. 6 *A*). Treatment with CTM (1.0 mg/kg i.v.) and THIO (10 mg/kg i.v.) attenuated the decrease in nasal cavity volume produced by compound 48/80 (Fig. 6 *A*). CTM (1.0 mg/kg i.v.) and THIO (10 mg/kg i.v.) given individually had no effect. Also, combined i.v. CTM and THIO treatment produced a 45% reversal of the effect of compound 48/80 on A_{min} values that was not statistically significant (Fig. 6 *B*).

Effect of Oral Histamine H_1/H_3 Receptor Antagonist Treatment on Nasal Cavity Volume

There was no difference between left and right baseline nasal volumes from control guinea pigs, 0.703 ± 0.097 and 0.669 ± 0.095 mL (n = 8), respectively. Baseline volume ratios between treatment groups were not different. Topical administration of compound 48/80 (1.0%) into the left nasal cavity of control animals decreased the nasal cavity volume (Fig. 7 *A*). Combined oral H_1/H_3 treatment with CTM (10 mg/kg) and THIO (30 mg/kg), but neither

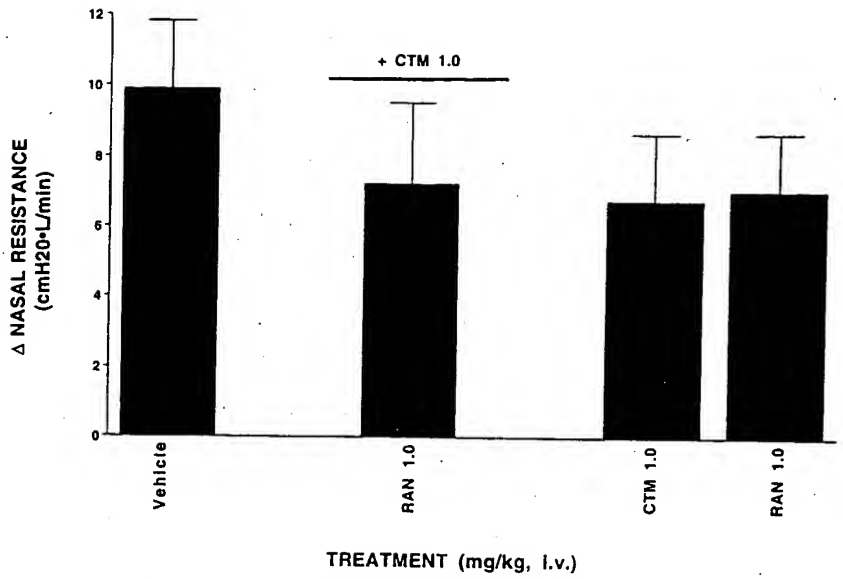

Figure 5. Effect of chlorpheniramine (CTM) and ranitidine (RAN) treatment on compound 48/80-induced increase in nasal airway resistance (NAR). Shown are the actions of CTM (1.0 mg/kg) combined with RAN (1.0 mg/kg), and CTM (1.0 mg/kg) and RAN (1.0 mg/kg) given alone compared to vehicle control animals on the increase in NAR due to exposure to aerosolized compound 48/80 (1%). Each bar represents the Mean ± SEM of 5–7 animals.

TABLE I

Effects of Histamine H1/H3 Antagonists on Blood Pressure and Nasal Airway Resistance

Treatment (mg/kg, i.v.)	Blood Pressure (mmHg)		Nasal Resistance (Δ cm H20·L/min)
	10 min*	25 min*	25 min*
Vehicle	129 ± 6	123 ± 7	10.6 ± 1.6
PPA (1)	174 ± 2#	140 ± 11	1.3 ± 0.9#
CTM (0.8) + THIO (10)	143 ± 13	119 ± 18	2.8 ± 2.2#
CTM (0.8) + CLOB (3)	113 ± 15	116 ± 12	3.4 ± 2.7#
Loratadine (3) + THIO (10)	108 ± 20	116 ± 13	2.1 ± 0.5#

**= Time after drug treatment.*
#Statistically different from control animals; $P < 0.05$.

drug given alone inhibited the decrease in nasal cavity volume produced by compound 48/80 (Fig. 7 *A*). PPA (10 mg/kg p.o.) also produced nasal decongestion. The maximum effect of compound 48/80 on A_{min} measurements is shown in Fig 7 *B*. Baseline left and right A_{min} values for control guinea pigs were 0.036 ± 0.004 and 0.036 ± 0.003 cm^2 and were not different between treatment groups. Topical administration of compound 48/80 into the left nasal cavity had no effect on contralateral A_{min} values from the right saline treated naris (data not shown). Both H_1/H_3 blockade with CTM (10 mg/kg p.o.) and THIO (30 mg/kg p.o.) and treatment with PPA (10 mg/kg p.o.) partially reversed the effects of compound 48/80 on A_{min}; however, these effects were not statistically significant. Treatment with CTM (10 mg/kg p.o.) or THIO (30 mg/kg p.o.) alone or together had no effect on systolic blood pressure, whereas PPA (10 mg/kg) produced an increase in blood pressure.

DISCUSSION

The present findings are the first demonstration that combined blockade of H_1 and H_3 receptors produces decongestant activity in an experimental model of nasal congestion. By evaluating changes in NAR produced by nasal exposure to compound 48/80 in the cat, we show that regardless of whether the H_1 antagonist was CTM or loratadine and the H_3 antagonist was THIO or CLOB, a synergistic decongestant effect was observed when the H_1 and H_3 antagonists were combined. The decongestant effect of combined H_1/H_3 blockade was equivalent to the decongestant effect of the α-agonist decongestant PPA. However, in

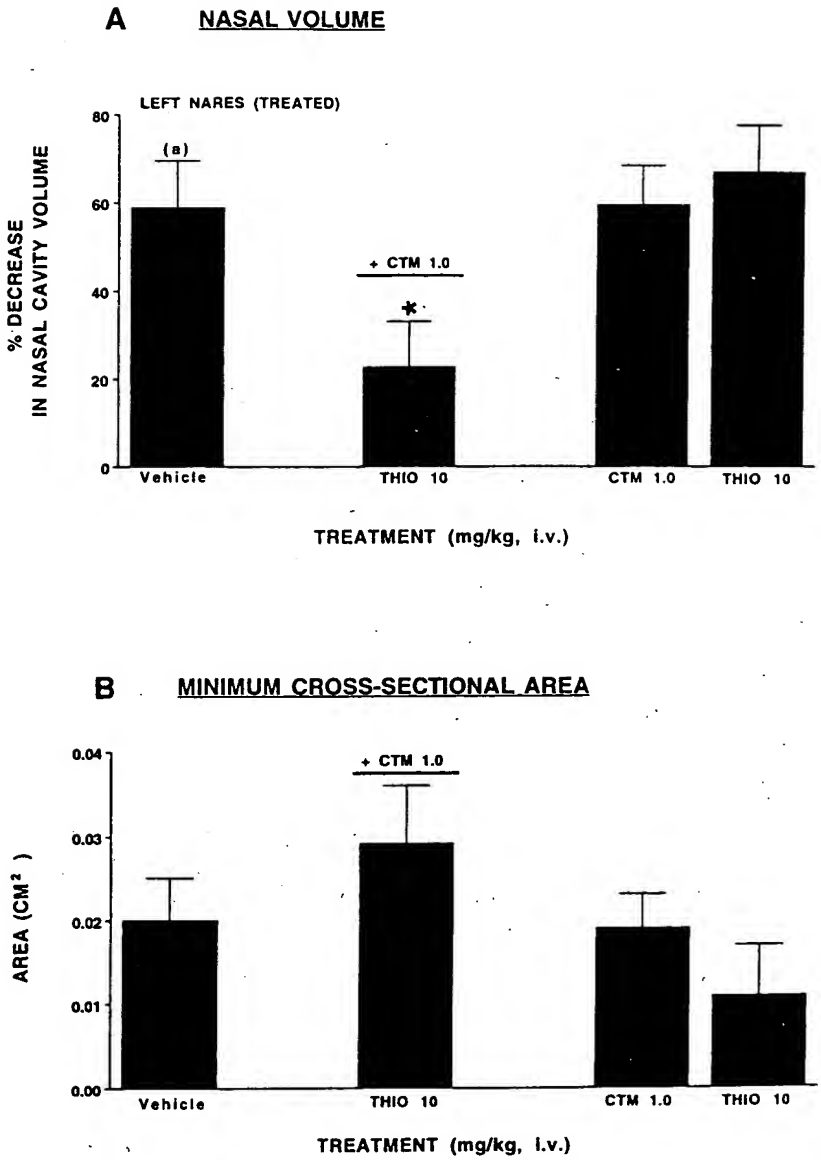

Figure 6. Effect of chlorpheniramine (CTM) and thioperamide (THIO) on compound 48/80-induced reduction in nasal cavity volume and minimum cross sectional area (A_{min}). The figure illustrates the actions of CTM (1.0 mg/kg i.v.) combined with THIO (10 mg/kg i.v.) on the decrease in (A) nasal cavity volume and (B) A_{min} due to exposure to topical compound 48/80 (1%). Each bar represents the Mean ± SEM of 4–8 animals ($p < 0.05$ compared to vehicle).*

contrast to PPA, which produces a significant hypertensive effect, the decongestant action of dual H_1/H_3 blockade did not affect systemic blood pressure.

A synergistic decongestive effect of combined H_1/H_3 treatment was also demonstrated against congestion induced by compound 48/80 when evaluated by AcR. AcR is a highly sensitive, noninvasive, and reliable technology increasingly used clinically to evaluate nasal patency.[21–24] In preclinical studies, this method has been demonstrated to be useful in assessing nasal patency in guinea pigs[25] and most recently in cats.[19] This technology allows evaluation of changes in the geometry (cross-section area) of nasal airways by means of sound reflection. With AcR, congestion of the nasal airways results in a decrease in the minimal cross-sectional area of the nasal cavity. This can be directly assessed by changes in the amplitude and volume of reflective acoustic waveform.[24] Using AcR, we observed that compound 48/80 produced nasal congestion evident by a decrease in nasal cavity volume and A_{min} values. The decreases in nasal cavity volume and A_{min} were blocked by pretreatment with PPA. In addition, our studies show that nasal decongestion results with i.v. and oral combined H_1/H_3 treatment. When given orally, the effect of combined CTM and THIO on nasal cavity volumes lasted 3 hours after dosing. Also observed with combined H_1/H_3 antagonist treatment was a partial reversal of the effects of compound 48/80 on A_{min}. This finding suggests a possible pharmacological action at the level of the nasal valve.[26] In a previous

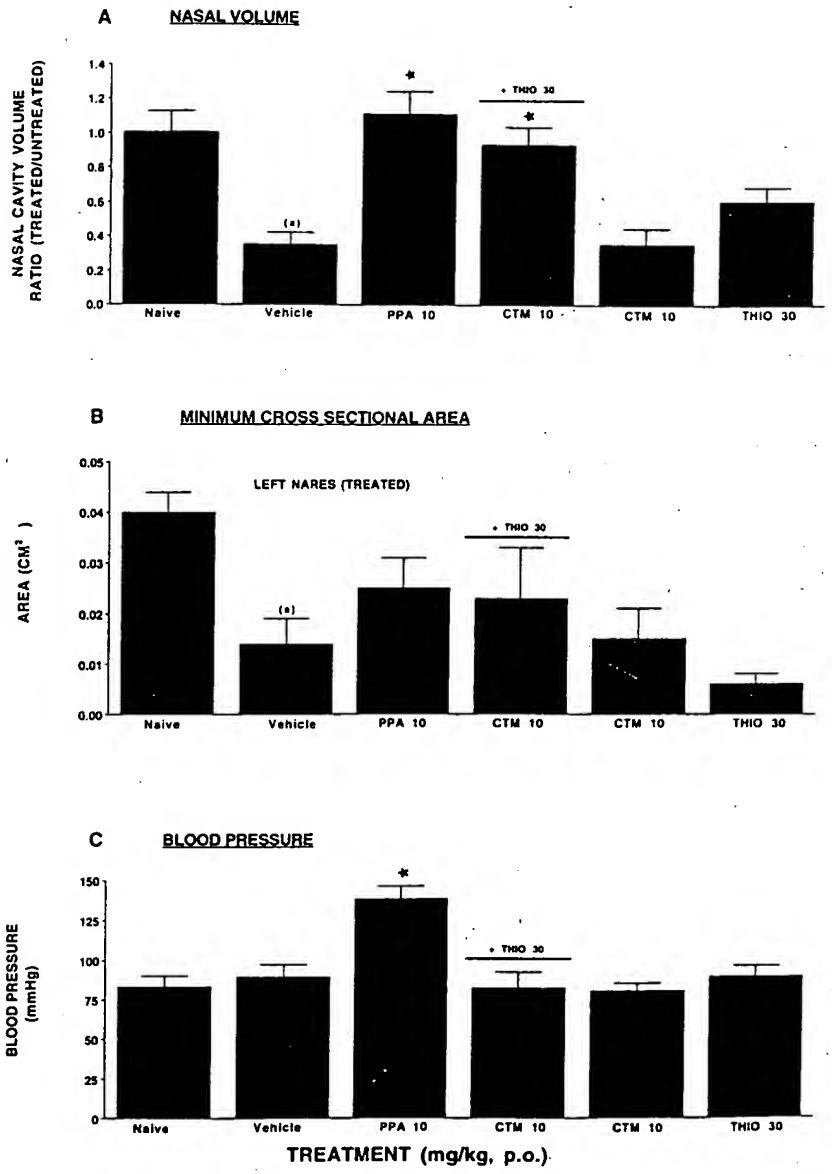

Figure 7. Effect of oral chlorpheniramine (CTM) and thioperamide (THIO) on compound 48/80-induced changes in nasal cavity volumes, minimum cross sectional areas (A_{min}), and systolic blood pressure. The actions of CTM (10 mg/kg p.o.) combined with THIO (30 mg/kg p.o.) and CTM (10 mg/kg p.o.), THIO (30 mg/kg p.o.) and PPA (10 mg/kg p.o.) given alone compared to vehicle control (compound 48/80 only) and naive animals (no treatment) on the decreases in (A) nasal cavity volume, (B) A_{min}, and (C) blood pressure due to exposure to topical compound 48/80 (1%). The volume data are expressed as the ratio of the volume of the left (treated) naris versus the right (untreated) naris. Each bar represents the Mean ± SEM of 5–8 animals ($p < 0.05$ compared to vehicle; (a) = $p < 0.05$ compared to naive animals receiving no treatment).*

study in which we characterized the decongestant actions of PPA using AcR, we also found that PPA reverses the effect of compound 48/80 on A_{min} measurements.[19] In humans, the nasal valve plays an important role in regulating NAR and may be an important pharmacological target for nasal decongestants. The nasal valve is located within the first few segments of the nasal cavity, and in humans the nasal valve has been shown to be the A_{min}.[27]

Evidence supporting an important physiologic role for histamine H_3 receptors in the regulation of nasal resistance is provided by the finding that H_3 receptor activation modulates sympathetic control of nasal blood flow and nasal resistance in the cat.[18,28] Based on these studies and the present results, it is proposed that activation of prejunctional histamine H_3 receptors by mast cell derived histamine, released by compound 48/80 or antigen cross-linking IgE molecules, may inhibit sympathetic outflow controlling vasodilatation and lead to nasal congestion. Blockade of both histamine H_1 and H_3 receptors may provide an additional benefit by blocking the H_3 vasodilation component that is

not blocked by H_1 antagonists (Fig. 1). This added activity appears to confer decongestant activity when H_1 antagonists and H_3 antagonists are given concomitantly. We propose that the decongestant activity of combined H_1/H_3 blockade, with a H_1 antagonist like loratadine and a selective H_3 antagonist, or with a novel drug with dual H_1/H_3 antagonist properties, may provide a novel approach for the treatment of allergic nasal congestion without the hypertensive liability of current therapies.

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